

2016

Synthetic Biology for Autotrophic and Heterotrophic Production of Ethanol

Nathanael Braselton

South Dakota State University

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SYNTHETIC BIOLOGY FOR AUTOTROPHIC AND HETEROTROPHIC
PRODUCTION OF ETHANOL

BY

NATHANAEL BRASELTON

A thesis submitted in partial fulfillment of the requirements for the

Master of Science

Major in Biological Sciences

Specialization in Microbiology

South Dakota State University

2016

SYNTHETIC BIOLOGY FOR AUTOTROPHIC AND HETEROTROPHIC
PRODUCTION OF ETHANOL

This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science in Biological Sciences degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidates are necessarily the conclusions of the major department.

Ruanbao Zhou, Ph.D.

Date

Thesis Advisor

Volker Brozel, ~~Ph.D.~~

Date

Head, Department of Biology & Microbiology

Dean, Graduate School

Date

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ABSTRACT

SYNTHETIC BIOLOGY FOR AUTOTROPHIC AND HETEROTROPHIC
PRODUCTION OF ETHANOL

NATHANAEL BRASELTON

2016

Growing energy demand and rising levels of greenhouse gases has put massive strain on the global environment. Alternatives to fossil fuels are being developed in an attempt to curb climate change. Biotechnology has made large strides in order to create a completely renewable energy source by genetically modifying microbes to produce biofuels and other “green” high value compounds. In this thesis project, (1) *E. coli* ATCC9637 (*E. coli* W) was genetically modified to produce bioethanol from beet juice which contains mainly sucrose. The ethanol productivity by engineered *E. coli* W was 18.8 mg/L/H/OD₆₀₀.

(2) Cyanobacterium *Anabaena* sp. PCC7120 was successfully engineered to produce and secrete biofuel ethanol using CO₂, water and sunlight.

(3) Another attempt in this study was made to increase production of bioethanol in ethanol-producing *Anabaena* strain by introducing a supplementary CO₂-fixing photorespiratory bypass pathway. Although Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) is responsible for the majority of carbon assimilation on Earth, RubisCO has poor specificity between CO₂ and O₂ which can lead to photorespiration and ultimately the loss of fixed carbon and nitrogen. The 3-

hydroxypropionate (3-HPA) bypass was introduced into ethanol-producing *Anabaena* strain in an attempt to increase CO₂-fixation and then increase ethanol production.

Introduction of the 3-HPA bypass into *Anabaena* showed only a marginal increase photosynthetic activity and a decrease in growth rate. Additional genetic manipulation would be required to alleviate bottlenecks and toxic intermediates in order to create a fully functioning supplementary CO₂-fixation pathway in *Anabaena* 7120.

Chapter 1: Introduction & Objectives

1.1 Introduction

At the dawn of the last century medical services, industrialization, and technology have vastly improved. Improvement of these services has allowed the global population to climb to unprecedented levels of 7.2 billion people. In addition, the population is expected to increase by another 2 billion by 2050 (United Nations 2014). The global population has put massive strain on the environment with increasing numbers of the population moving to the cities. The migration towards cities has increased the standard of living and increased the demand for greenhouse intensive food such as dairy and meat products. These products require more land, water, and minerals which are a finite resource. These issues are compounded by the fact that the environment is changing and has already caused complications for food production in some areas (Garnett, 2014).

Currently energy needs are being met primarily by fossil fuels. Fossil fuel production is currently producing 27 billion barrels per year. This production does not include other fossil fuel extraction such as shale oil or natural gas. Fossil fuels which are a finite resource will at some point hit a maximum production output termed as “peak oil”. The potential catastrophic situation that is created from this dependence on fossil fuels comes after peak oil production in which, consumption of fossil fuels will outweigh production causing an energy crisis. If the current trend of using fossil fuels as a primary source of energy stay the same GDP growth could be affected as oil consumption and GDP growth are currently highly correlated. It would be prudent to consider other implications of

using fossil fuels as fossil fuels emit carbon dioxide into the environment which has caused global temperatures to rise (Brecha, 2013).

1.2 Objectives

Fossil fuel depletion and environment degradation (due to elevated CO₂ emissions) are the two pressing global problems. To alleviate the pressing problems, sustainable and renewable bio-energy sources must be developed to replace petroleum-derived transportation fuels. This research is to metabolically engineer microorganisms to produce fuel ethanol using renewable feedstocks. The two specific aims are:

- 1) Metabolically engineer *E. coli* W to directly produce fuel ethanol using renewable beet juices
- 2) Metabolically engineer cyanobacteria to directly produce fuel ethanol using water, CO₂ and sunlight

Chapter 2: Literature Review

2.1 First-generation biofuels

The general public is becoming more aware of the drawbacks of fossil fuels as a finite resource. As these concerns grow there has been a large push in biotechnology to create biofuels which use CO₂ to produce biomass that can be turned into a fuel or energy source. The sequestering of atmospheric CO₂ into a biomass for use as a biofuel will allow for a renewable resource that is carbon neutral. The production of biofuels such as bioethanol or biodiesel from plant biomass has garnered much interest in past few decades and led to the advent of “first generation” biofuels.

First generation biofuels primarily relate to biofuels which come from easily-digested feedstocks such as starch from corn or sucrose from sugar cane as well as vegetable oils. These feedstock crops are either chemically treated or biologically degraded. The processing of the feedstock varies by both the type of feedstock and the type of biofuel desired. Biofuels created from starch or sucrose typically are fermented using yeast which converts the sugars to biofuel ethanol. Alternatively, biofuels from vegetable oils typically are processed by a transesterification process so the fuel can be used in a typical combustion engines.

2.1.1 Feedstocks from starch & sugar

Biofuel created from starch and sucrose has been in existence for many years. Bioethanol can be produced from a variety of feedstocks which include primarily corn or soybeans in US production, sugarcane in Brazil, and wheat in Northwest Europe (de Vries, van de Ven, van Ittersum, & Giller, 2010). The two most dominating countries in

global ethanol production are the US at 58% and Brazil with 28% as of 2015 (Figure 1). Biofuel production from feedstocks increased rapidly since 1980 production started at 4.4 billion liters and increased to 50.1 billion liters by 2005 (Nigam & Singh, 2011).

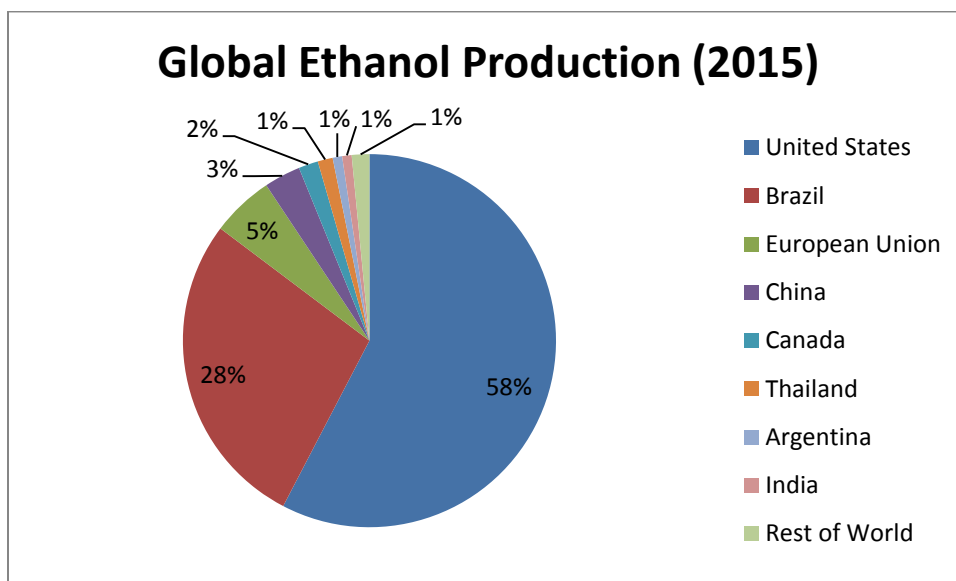


Figure 1: Global ethanol production in 2015, adapted from Renewable fuels association (Renewable Fuels Association, 2015).

Biofuel production processes can vary widely depending on the feedstock from which it was produced. Brazil's biofuel production focuses mainly on sugarcane which is easier to process. Sugarcane is easier to process because after the sugar has been separated from the cane it can be directly fermented by yeast into ethanol. Ethanol from sugarcane as a first generation biofuel is easier to process and produce than corn based ethanol (Martin & Grossmann, 2013).

Biofuel production from corn is separated into two different methods of wet milling or dry grinding processes. Wet milling was introduced first since the ethanol production was a by-product from high fructose corn syrup production which started

around 1974. Currently wet milling production of ethanol only contributes to 5% of the total ethanol production in the US. The majority of ethanol production is derived from dry-grinding process which incorporates 95% of the ethanol production in the US. Dry milling process of ethanol production is lengthy which requires stages of grinding, scarification, fermentation, centrifugation, and drying. In this dry milling process, Corn is first grinded into a fine meal consistency, water is then added along with various enzymes to break down the starch into a mash (single sugars). The resulting mash is then fermented and followed by distillation. After distillation of ethanol is completed, the resulting product is an azeotropic mixture of 95% ethanol with 5% water (%w/w). In order for ethanol to be blended with gasoline as a biofuel it must reach near 100% purity. To further purify the ethanol from 95% to 100% the use of molecular sieves are required (Martin & Grossmann, 2013).

2.1.2 Feedstocks from oilseeds

Biodiesel production has been rapidly growing in the US. In 2008 biodiesel produced 20 million barrels of biodiesel by 2013. Another large biofuel producing country is Brazil, which is the second largest producer of soybeans globally and 85% of the soybean oil is used in biodiesel production. In addition, the conversion of soybean oil to biofuel production in Brazil is sustainable and helps reduce greenhouse gas emission and utilizes low-carbon agriculture (Caldeira-Pires et al., 2013).

Production of biodiesel from oilseeds can be accomplished using a variety of vegetable oils which include sunflower, soybean oil, rapeseed oil, palm oil, cottonseed oil, canola oil, and peanut oil. While the specific requirements of producing biodiesel can

change depending on the oilseed used typically the production entails transesterification of triglycerides with alcohols such as methanol and ethanol. A base catalyst is required as well to produce the fatty acid alkyl esters and glycerol. Various different methods have been put to use in the processing steps to produce biodiesel from oilseeds.

Some of the best processes for biodiesel production include heterogeneous catalyzed processes (enzymatic, basic or acid based) or supercritical based processes. These processes were determined more efficient because they allow for fewer separation stages and better competitive pricing per liter (Martin & Grossmann, 2013).

2.1.3 Disadvantages of first-generation biofuels

Although biofuel production has greatly increased there are still concerns regarding the disadvantages which include: land use, conversions of grasslands to farmland, carbon debt and greenhouse gas emissions from the production of first generation biofuels.

Major concerns of land use are due to the use of farmland to farm biofuel crops over preference of crops for food. Ethanol production used 20% of the corn crop production in 2007 which is a substantial increase from 5% used in 2000. The use of corn crops for biofuel production creates a food vs fuel competition, which has forced farmers to convert uncultivated land into farmland in order to meet demands (Odling-Smee, 2007).

The increasing demand for the production of biofuels has caused previous grassland to be converted to croplands to try and meet demands of biofuel consumption in the US. Conversion rates of grassland to corn or soybean farmland has reached 1-5.4%

annually and is only increasing as production of biofuels increases every year. This conversion of grasslands to corn or soybean crops raises many concerns as the land that is being converted has high risk of soil erosion and drought vulnerability. In addition, converted grass lands are in very close proximity to the wetland breeding lands for waterfowl and could greatly impact the local ecosystem (Wright & Wimberly, 2013).

Another major concern of the conversion of grasslands to farmland is the carbon debt. This carbon debt created from conversions of grasslands to cropland is estimated to take anywhere from 3 to 50 years to make up for the conversion of grasslands, rainforests, savannah, and peat lands to farmland to offset the net gain of producing biofuels (Gelfand et al., 2011).

In relation to the carbon debt, another major concern is the greenhouse gas (GHG) emissions needed to farm these crops. These carbon dioxide emissions from farming to end product bioethanol are estimated to be 17 to 420 times more than the annual GHG reductions provided by the use of the biofuels themselves (Fargione, Hill, Tilman, Polasky, & Hawthorne, 2008). It would likely be more prudent to instead use the waste biomass or biomass farmed from abandoned agriculture land.

These concerns of land use, land conversion, greenhouse gas emissions, and carbon debt all create serious concerns if first-generation biofuels are truly sustainable for biofuel production. Other alternatives should be explored that can solve the food vs fuel problem that don't increase the carbon debt. Considerations must be made so that a sustainable fuel platform can be created that takes into account greenhouse gas emissions and land conversion and does not harm local ecosystems.

2.2 Second-generation biofuels

When relating to biofuels the term “plant biomass” refers to plant matter such as fibrous non-food plant material which contributes to the majority of the plant. This includes up to 75% of the plant material which is composed of cell wall components such as lignin, cellulose, and hemi-cellulose. These cell wall products are composed of polysaccharides which are abundant in the non-edible parts of the plant. In wheat (*Triticum aestivum*) the stems and other non-edible parts of the plant can incorporate just as many sugars as the starch from the grains themselves (Gomez, Steele-King, & McQueen-Mason, 2008) . The two most common polysaccharides involved in plant cell walls include cellulose and hemicellulose which could be used in the production of biofuels (Pauly & Keegstra, 2008). These sugars can be broken down using hydrolysis and fermentation or gasification in order to produce advanced biofuels. Ethanol from first-generation biofuels must be blended with gasoline for regular vehicles or used as full substitute in flex-fuel vehicles. Second generation biofuels can be created using processes such as Fischer–Tropsch diesel (FT-diesel) or BTL (Biomass-to-Liquids) as a complete replacement or even substitution for diesel (Naik, Goud, Rout, & Dalai, 2010). This large pool of potential sugars from the non-edible portions of the plant could side-step the issue of the food vs fuel dilemma that is presented by first-generation biofuels (Figure 2). In addition, another boon of second generation biofuels is that the products such as Bio-DME (Dimethyl Ether) that can be used as a full replacement as a fuel source with only minor adaptations to engines (Naik et al., 2010).

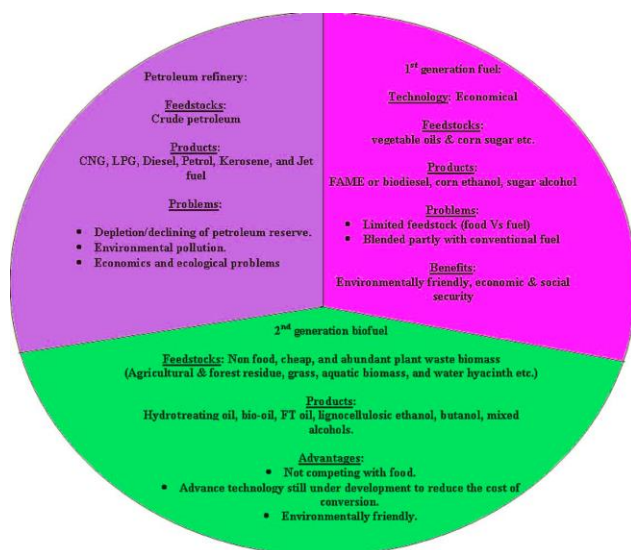


Figure 2: Comparison of first and second generation biofuels and petroleum (Naik et al., 2010).

2.2.1 Process steps to generate biofuel from lignocelluloses

The two main avenues to produce biofuels from plant biomass include thermochemical or biochemical processing (Figure 3). Thermochemical processing refers to processing the plant biomass by heating in varying concentrations of oxygen. Heating the biomass in the absence of oxygen is termed pyrolysis which can produce a variety of organic liquids that are then processed into biofuels. Another process called gasification occurs when the plant biomass is heat treated with low concentrations of oxygen which produce hydrogen or syngas. The products from gasification can then be converted to liquid biofuels using the Fischer-Tropsch method.

Biochemical processing uses lignocellulose and converts it to sugars which then are fermented into alcohol similar to ethanol production from first-generation biofuels. Biochemical processing appears to be more advantageous than thermochemical

processing because of lower startup and maintenance costs. Biochemical processing also allows for smaller processing facilities than thermochemical processing. Biochemical processing occurs in two main steps. First, plant mass is hydrolyzed by either treating the plant biomass with acid such as sulfuric acid or hydrochloric acid. Alternatively, the biomass is pretreated usually with the use of diluted acids and then enzymatically digested using a cocktail of different enzymes produce simple sugars from cellulose (Gomez et al., 2008).

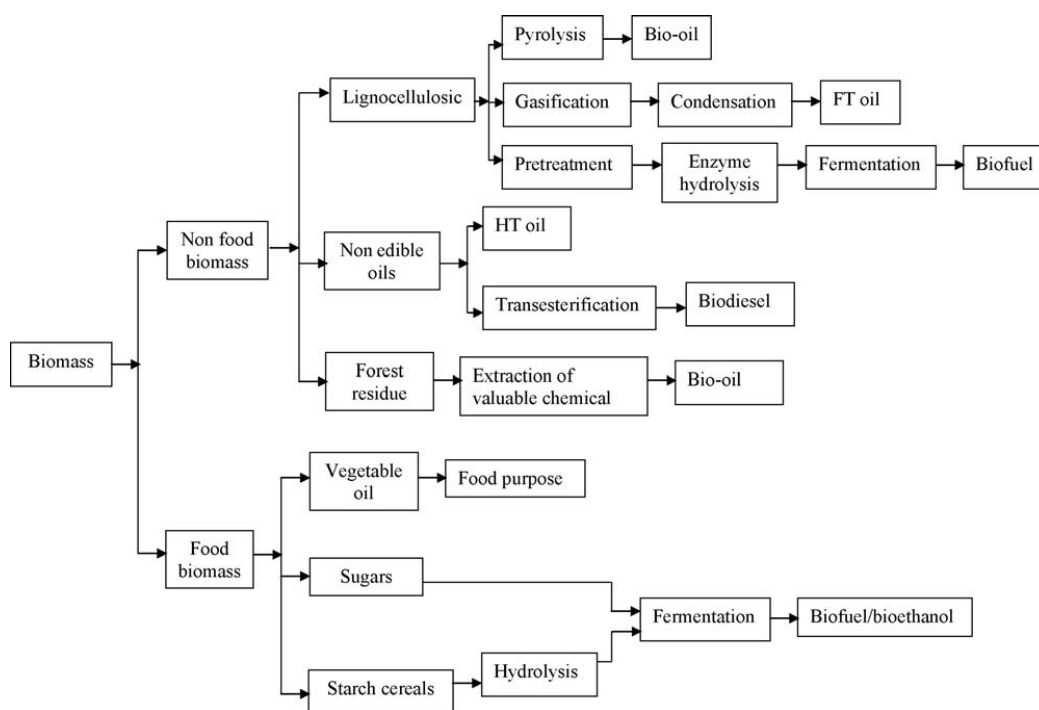


Figure 3: Production of Second generation biofuels from biomass (Adapted from (Naik et al., 2010))

2.2.2 Disadvantages of second-generation biofuels

Even though more efficient methods have been created in the production of second generation biofuels the process is still not yet cost-effective (Naik et al., 2010). The largest technical barrier in the production of second generation biofuels is the saccharification processing step in biochemical processing. Saccharification is the major

technical bottleneck because plant cell walls have evolved to have a strong plant structure to allow for growth of the plant and is resistant to biochemical attacks by other organisms. These two properties are important for the organism because without resilience to pests and pathogens the plant would not thrive well in its native environment. The rigid structure and protection to biochemical attacks is what causes the resistance to enzymatic digestion required to release the sugars in the plant cell walls (Wingren, Galbe, & Zacchi, 2003).

Even though biochemical processing has developed more efficient solutions to digesting the plant cell wall material there are still problems regarding cost efficiency. Previously, plant biomass was enzymatically digested using costly enzymes. However, research performed recently by Novozymes and Genencor funded by the US Department of Energy has allowed for the cost of these enzymes to be reduced ten-fold (Zhang, Himmel, & Mielenz, 2006). However, due to the nature of biochemical processing using digestive enzymes a pretreatment process is required in order for the enzymes to properly digest the cell wall material into simple sugars. Although, there has been a reduction in prices of the digestive enzymes there are still cost-efficiency problems due to the expensive pretreatment of the plant cell wall material (Gomez et al., 2008).

2.3 Third-generation biofuels

The development and production of first and second generation biofuels has provided hope for reducing the carbon footprint in order to meet energy demands. However, the production and efficiency has yet to reach sustainable levels that can reach carbon neutral levels. Taking these criticisms into account it is advantageous to look into

other avenues for biofuel production besides using plants as a source for biofuel production. A similar model for creating biofuel from CO₂ in the atmosphere would be photosynthetic organisms such as microalgae and cyanobacteria. Cyanobacteria and microalgae like plants engage in oxygenic photosynthesis which allows the organisms to use light and fix CO₂ to produce biomass. Not only do these organisms have the capability to use photosynthesis but can be cultured for production of other products such as lipids, carbohydrates, biogases, liquid biofuels, and even animal feed stocks (N. K. Singh & Dhar, 2011). The use of algal biomass for the conversion to biofuels is termed “third generation biofuels”. Third generation biofuel production aims for a more sustainable process of generating biofuels from sequestering carbon from the atmosphere and converting it into biomass

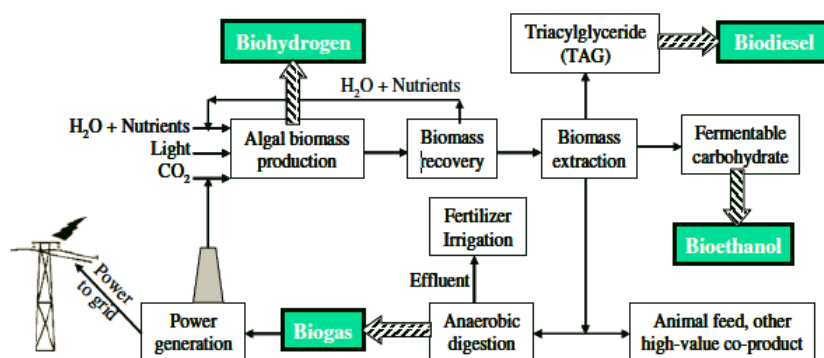


Figure 4: Model for high-value products derived from Algal mass production (N. K. Singh & Dhar, 2011).

2.3.1 Background on photosynthetic microorganisms

Aquatic microbial oxygenic photoautotrophs or AMOPS are a subset of microorganisms that includes organisms such as, cyanobacteria, diatoms and microalgae. AMOPS are advantageous for the production of sustainable resources due to their ability to produce high value compounds by using energy from light and to fix carbon from the atmosphere (Menetrez, 2012). Microalgae have historically been used for human health and food products as well as a feedstock for fish and livestock dating back to ancient times (N. K. Singh & Dhar, 2011). AMOPS are versatile due to their ability to grow in a variety of places such as brackish sea water and even in wastewater (Menetrez, 2012). In addition, AMOPS have a relatively fast generation time of one to three doublings per day depending on the species. AMOPS do not have specialized organs such as leaves, stems, and roots and other specialized structures common in plants. However, AMOPS are able to photosynthesize and contain chlorophyll and photosynthetic pigments. All AMOPS contain green chlorophyll and other photosynthetic pigments depending on the species type which may include, red, blue, gold, and brown pigments. Algae, particularly cyanobacteria are very old organisms that have fossil records dating back to around 3 billion years during the Precambrian era (Menetrez, 2012). Algae were the original pioneers of photosynthesis and produced much of the oxygen in atmosphere today. Algae contribute a large quantity of the ancient organic carbon, such as coal and petroleum. Currently, the most abundant AMOPS are single-cell drifters phytoplankton which are able to grow rapidly and have a much higher photosynthetic efficiency than that of land-based plants (N. K. Singh & Dhar, 2011). AMOPS are prolific and ubiquitous within the biosphere and can thrive both within freshwater and saltwater environments.(Menetrez, 2012).

2.3.2 *Advantages of third-generation biofuels*

Using AMOPS for the production of biofuels has many advantages over using plants. AMOPS have fairly fast growing rates typically within 1-3 doublings per day which allows for faster production than from plant based biofuels (Menetrez, 2012). In addition to faster growth rates, AMOPS also are much more efficient in photosynthesis than plants by utilizing 10% of total sunlight which can be converted it into biomass. In contrast, typical land based plants only utilize about 1% of total sunlight. AMOPS are also capable of growing in brackish water or saltwater which does not require arable land. This allows for AMOPS to be cultivated on land not suitable for food crops and removes the issue of food vs fuel. AMOPS also have a large makeup of lipids (20-50% dry weight) and large content of carbohydrates (20-50% dry weight) that can be fermented into biofuels. Because AMOPS are tolerant to some unique environments it has been demonstrated that these organisms are able to grow in wastewater and convert CO₂ into biomass using light as an energy source (Udom et al., 2013). AMOPS growing in wastewater provides additional benefits as the organisms can use the nitrogen and phosphorus present in the wastewater for nutrients and do not require potable water while simultaneously recycling CO₂ which would otherwise be released into the atmosphere (Udom et al., 2013). Previous studies have also predicted that oil extracted from microalgae (30% oil by wt) can yield 58,700 liters per hectare (l/ha), while the closest competitor for plant based oils was oil palm with only 5,950 (l/ha), 10-fold less (Table 1).

Table 1: Oil yields from the various biomass sources (J. Singh & Cu, 2010)

S.N.	Crop	Oil yield (l/ha)
1	Corn	172
2	Soybean	446
3	Peanut	1,059
4	Canola	1,190
5	Rapeseed	1,190
6	Jatropha	1,892
7	Karanj (<i>Pongamia pinnata</i>)	2,590
8	Coconut	2,689
9	Oil palm	5,950
10	Microalgae (70% oil by wt.)	136,900
11	Microalgae (30% oil by wt.)	58,700

2.3.3 Environmental conditions for cultivation

Cultivation of cyanobacteria is successful when the proper environmental conditions have been achieved. The environmental conditions such as light intensity and wavelength, nutrient composition of the media, gas composition, temperature, and aeration can vary depending on the species being cultivated. To achieve highest rates of biomass production for biofuel production the optimum cultural conditions need to be met.

2.3.3.1 Lighting Conditions

Microalgae have to balance the absorption of the excitation energy present in sunlight with the production of chemical energy and reducing agents in order to properly grow and perform cell maintenance. Microalgae utilize sunlight and atmospheric carbon (CO₂) to create chemical energy (ATP) and reducing agents (NADPH) needed for cell functions and growth. Failure to balance this process can lead to excess excitation which produces reactive oxygen species (ROS) that promote photo-inhibition and interferes with normal cell functions which can ultimately result in photo-oxidative death.

Microalgae utilize photosynthetic active radiation (PAR) from sunlight in the 400-700nm wavelength range to split H₂O to produce O₂, hydrogen ions and electrons. The hydrogen ions are then used to power a transmembrane chemiosmotic potential which allows for the formation of ATP and NADPH required for cell functions. The light absorption in microalgae occurs in the light-harvesting antenna structures located on the thylakoid membranes. Typically light absorption is considered fully saturated around 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ which is only 10% of the full sunlight intensity observed (2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$) during the summer months. In addition, microalgae on the surface of water is under constant saturation of light while microalgae deeper in the water suffers from lower light conditions due to self-shading caused by the microalgae closer to the surface. Typically self-shading problems can be addressed by shortening the light path length so that the light is more evenly distributed. Proper mixing and/or aeration can also alleviate self-shading problems for microalgae cultured in specially designed photo-bioreactors (Torzillo, Pushparaj, Masojidek, & Vonshak, 2003).

2.3.3.2 *Nutrient Composition*

Microalgae require rather simple nutrients for growth which typically include: macronutrients, minerals, and trace microelements depending on the microalgae species. The two main nutrients that are required by microalga growth are nitrogen and phosphorus at a typical ratio of 16N: 1P. However, in most cases nutrients are added in excess to ensure that nutrient limitation does not inhibit growth. Nutrient composition can vary widely even when growing the same microalgae culture and dependent on the end use of the culture (N. K. Singh & Dhar, 2011). The supply of nitrogen to the culture is crucial to microalgae that are cultivated for their triacylglycerides (TAGs) which are

typically used as a feedstock for biodiesel. It was stoichiometric calculated that if an oil rich microalgae contained 20% TAG by weight, then for every 1 kg of TAG feedstock produced 0.36 kg of nitrogen (0.46 kg NH_4^+ or 1.6 kg NO_3^-) would be required (Peccia, Haznedaroglu, Gutierrez, & Zimmerman, 2013). Trace microelement requirements can vary depending on species of microalgae but typically trace metal ions such as copper, cobalt, zinc, magnesium, iron, molybdenum, and nickel are critical for some photosynthetic processes. Previously stated trace metal ions are thought to aid in nitrogenase activity and thought to aid in the production of hydrogen gas (N. K. Singh & Dhar, 2011).

2.3.3.3 Gas Composition

Proper growth conditions of microalgae require a large amount of carbon since 45%-50% of algae biomass is composed of carbon. It has been theoretically calculated that for every 1 gram of dry weight algae biomass produced requires 1.65-1.83 grams of CO_2 (Jiří Doucha, Straka, & Lívanský, 2005). The composition of the atmosphere is only 0.033% CO_2 and as such does not provide near the optimum amount of CO_2 for growth of microalgae cultures (N. K. Singh & Dhar, 2011). Supplying additional CO_2 to cultures allows for lowering the impact of CO_2 on the environment by sequestering the atmospheric CO_2 into biomass while simultaneously reducing production cost (Jiří Doucha et al., 2005). Typically CO_2 is blended with air into microalgae cultures by gas exchange vessels in photo-bioreactor and sumps in open raceway ponds. Oxygen released from microalgae cultures is also of concern because if oxygen levels exceed atmospheric oxygen levels photo-oxidation occurs which damages chlorophyll reaction centers. Photo-oxidation inhibits photosynthesis rates and lowers total production of biomass

(Molina, Fernandez, Acien, & Chisti, 2001). Photo-oxidation typically is not a concern in open air algae systems but needs to be closely monitored in closed photo-bioreactor where proper gas exchange is balanced between the culture and the atmosphere.

2.3.3.4 Temperature

Temperature is another important parameter in optimizing growth of microalgae. Typically higher temperatures will yield better growth rates until the optimum temperature is reached. Normal growth temperatures for microalgae typically fall between 15-35°C depending on the species (Chisti, 2007; Olaizola, 2000). Temperatures above optimal temperature start to induce stress on microalgae cultures and could potentially result in culture death. Temperatures below optimal levels will not typically cause death but will limit growth rate and consequently overall biomass production. Changing atmospheric temperatures due to diurnal cycles can effect biomass production particularly in open pond systems where night time temperatures can dip to 16°C and peak at 34°C during the midday (Olaizola, 2000). In raceway systems, a type of open pond system temperature control is mainly controlled by evaporation. Water loss from evaporation can be significant in these systems. During night cycles in open pond systems as much as 25% of the biomass can be lost due to respiration. The magnitude of this loss is dependent on the amount of light during the day, growth temperature, and the temperature during the night cycle. In contrast, closed photo-bioreactor systems allow for better temperature control which allows for the cooling of these systems during the night cycle which can reduce biomass loss from respiration (Chisti, 2007).

2.3.3.5 Agitation

Proper agitation in microalgae allows for better distribution of nutrients and gases in the cultures. Agitation helps more evenly distribute cells throughout the culture medium and prevent sedimentation. Agitation is performed through the use of mechanical agitation with pumps or paddlewheels primarily used in open pond systems. Other agitation methods use bubbling of gases typically through a sparger that is common in closed photo-bioreactors. In both types of systems the use of baffles can be beneficial to improve agitation or reduce vortex in bioreactors. Typically if the culture is bubbled with a gas mixture it is supplied externally as microalgae cultures grow better with a higher amount of CO₂ than usually present in the atmosphere (R. N. Singh & Sharma, 2012).

2.3.4 Cultivation methods

Numerous different cultivation methods exist for the growth microalgae cultures. The selection of cultivation method will typically have the most impact on total efficiency and final productivity of biomass production (Carvalho, Meireles, & Malcata, 2006). The methods will be divided into two main categories of open pond systems or closed photo-bioreactors (PBR). Open pond systems consist of outdoor system in which an area is excavated and then the culture is grown in the water filled area. PBR systems vary widely but typically consist of an enclosed system in which temperature, gas exchange, and agitation are tightly controlled.

2.3.4.1 *Open ponds*

Historically open pond systems have been the most used cultivation system and have been in use since the 1950's (Borowitzka, 1999; Brennan & Owende, 2010). Open pond systems include natural cultivation systems such as lakes, lagoons, and ponds. Open pond systems are typically constructed by excavating an area at a depth of 20 to 30 cm deep and can be as deep as 50 cm in unmixed ponds. These systems can either be lined using plastic or concrete or compacted earth can also be used (Brennan & Owende, 2010; Rawat, Kumar, Mutanda, & Bux, 2013). Above ground open pond systems with walls can also be constructed as long as impermeable materials are used (Chisti, 2007).

Four main types of open pond systems are used which include: 1) circular ponds, 2) raceway ponds, 3) thin layer, inclined ponds, and 4) unmixed ponds (Borowitzka, 2005). Circular ponds are composed of a central rotating arm that mixes the culture (similar to wastewater treatment plants) and productivities between $8.5 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$ to $21 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$ (N. K. Singh & Dhar, 2011). Raceway ponds are comprised of a closed loop and mixed with the use of a paddle wheel which are constructed in an oval shape and are the most used artificial cultivation system and typically provide the highest productivities (Figure 5) (Brennan & Owende, 2010; Jimenez, Cossio, Labella, & Niell, 2003). Thin layer, inclined ponds consist of small inclined shallow trays which have productivities of $31 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$ (J. Doucha & Lívanský, 2006; N. K. Singh & Dhar, 2011). Unmixed ponds have the lowest productivity (less than $1 \text{ g} \cdot \text{m}^{-2} \cdot \text{d}^{-1}$) and only a few different species of microalgae are suitable for this system (Benemann & Oswald, 1996; Jiří Doucha et al., 2005). It should be noted however, that these open pond systems are restricted to fast

growing algae species that are able to tolerate sub-optimal environmental conditions such as *Chlorella*, *Dunaliella*, and *Spirulina* (Chisti, 2007).

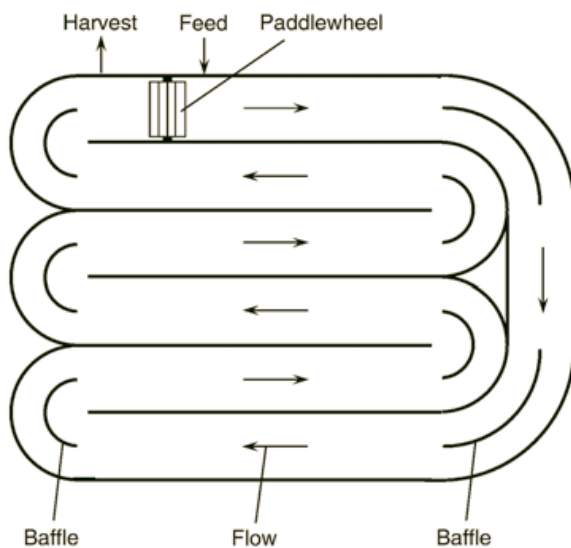


Figure 5: Aerial view of typical raceway pond (Chisti, 2007).

2.3.4.2 Closed photobioreactors

Open pond systems while viable still have a variety of problems associated with being open air which include, environmental changes, contamination, water evaporation, and low productivities (Chisti, 2007). These technical difficulties have led to the development of PBR systems which allow for numerous advantages. Some of the main advantages of PBR systems are due to the closed loop system which allows for better control of growth parameters such as temperature, gas composition, pH, and light concentration (R. N. Singh & Sharma, 2012). One of the largest benefits of PBR systems is the significant reduction of contamination which allows for the production of high-value compounds used in pharmaceutical and cosmetic industries (Ugwu, Aoyagi, & Uchiyama, 2008). In addition, due to lower contamination issues PBR systems allow for

the cultivation of monocultures (Chisti, 2007). The operating cost of PBR systems is much higher than open pond systems. However, PBR systems provide higher cell mass productivity, lower harvesting costs, and better control of contamination make it an attractive option (Carvalho et al., 2006). PBR systems have a large variety of designs which include bubble columns, flat plate reactors, helical reactors, tubular (horizontal and vertical) reactors (Figure 6), or combinations of different design types (Brennan & Owende, 2010; Chisti, 2007; R. N. Singh & Sharma, 2012).

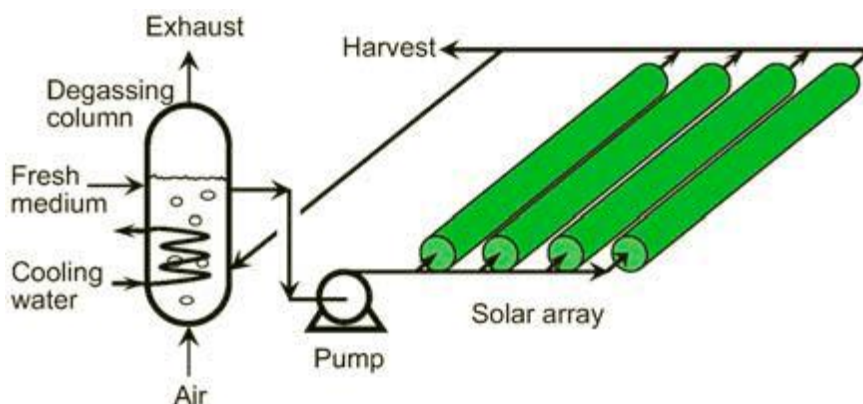


Figure 6: horizontal tubular PBR design (Chisti, 2007).

2.3.5 Disadvantages of third-generation biofuels

Energy production from third generation biofuels from cultivation of microalgae is theoretically possible there are still limitations that require continued improvement. One of the largest drawbacks of algae based biofuel is the processing steps required to create the end product. Large energy requirements are needed for agitation, harvesting, and drying of algal biomass before extraction. The energy requirements are compounded when scaling these systems to industry levels. This creates an environment in which third generation biofuels becomes non-competitive with other non-renewable energy sources

(N. K. Singh & Dhar, 2011). In addition, the productivity of these methods is dependent on environmental factors such as temperature, water availability, light intensity, pH, and gas composition. While many of these factors can be addressed with PBR systems, the efficiencies of third generation biofuels need to fully optimize in order to be fully competitive with non-renewable resources.

2.4 Fourth-generation biofuels

The development of first, second, and third generation biofuels have all focused on the production of renewable feedstocks. However, these renewable feedstocks need additional processing in order to create usable energy products. The processing steps for first through third generation biofuels include processes such as hydrolysis, fermentation, and transesterification which require large energy input in order to create biofuels. The energy requirements needed for these processes fail to make these generations of biofuel cost-competitive with fossil fuels. A system that allows for direct conversion of sunlight and CO₂ into usable products would provide a large advantage over previous generations of biofuel production. The most crucial part of fourth generation biofuels is the elimination of additional processing steps that would allow for direct recovery of the energy product which is secreted from the cells (Figure 7). This system would effectively make these microalgae species “cell factories” that are able to use sunlight and CO₂ and directly convert these inputs into usable “drop in biofuels” (complete compatibility with traditional petroleum-based fuels that can be interchangeable for conventional fuel) (Lu, Sheahan, & Fu, 2011). The elimination of processing steps from previous generations of biofuels and the ability to harvest the end products without destroying the culture would greatly reduce production costs.

Cyanobacteria have become a large research focus in the field of bioenergy and biofuels. Cyanobacteria strains have been genetically engineered to act as “cell factories” in which biofuels and other high-value compounds are continually secreted. This combination of biofuel development strategies with genetic engineering of cyanobacteria has led to the development of “Fourth generation” of biofuels (Lu et al., 2011).

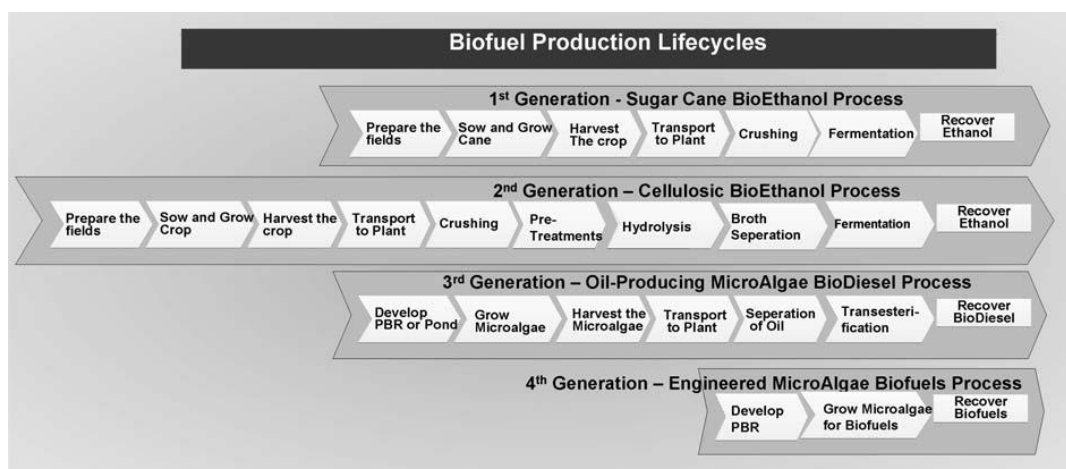


Figure 7: Contrast of processing steps for various generations of biofuel production (Lu et al., 2011).

2.4.1 Cyanobacteria: a model host for fourth generation biofuels

Cyanobacteria is an advantageous host for the development of fourth generation biofuels due to the relatively low nutrient requirements of CO₂, H₂O, and light and faster growth rate in comparison to plants. The term “Green Microbe” will be used to refer to two main subgroups of plant-like photosynthetic microorganisms which include cyanobacteria (Prokaryote) and microalgae (Eukaryote). These species of cyanobacteria are ubiquitous in nature and have been isolated from fresh water, saltwater, and soil. Many species of cyanobacteria have also been isolated from much more extreme

thermophilic and halophilic environments (Lu et al., 2011). Cyanobacteria are ancient microorganisms and some of the oldest forms of life dating back to 3.5 billion years ago (P. G. Falkowski et al., 2004). Cyanobacteria contribute 50% of CO₂ sequestration and 50% of O₂ genesis on earth (P. Falkowski, Raven, & Sakshaug, 1997). Cyanobacteria are thought to be the architects of the modern atmosphere and the first to develop oxygenic photosynthesis. Since cyanobacteria are classified as eubacteria the genetic structure is comprised of circular chromosome(s) which is much easier for genetic manipulation. The simple genetic structure is crucial because it allows for easier metabolic engineering which is required to modify cyanobacteria to produce biofuel.

Due to the development of accurate DNA sequencing methods and cost reduction in the last few decades numerous genomes of cyanobacteria species (≈ 320) have been fully sequenced. A few of them are listed in Table 2, and much more can be found in CyanoBase that covers the sequence of 39 strains of cyanobacteria. In addition to CyanoBase, more than 281 cyanobacterial genome sequences are currently available (Johnson, Gibbons, Gu, Zhou, & Gibbons, 2016). The genome sequencing of cyanobacteria species allows researchers to understand the genes and proteins involved in the metabolic cycles of these species. Since these genomes have been sequenced it allows for greater understanding of the biosynthesis pathways of most components of the cyanobacteria. Understanding of metabolic pathways of cyanobacteria species allows researchers to modify these metabolic pathways in order to manipulate cyanobacteria into “cell factories” for biofuels or other high-value compounds.

Table 2: Model strains of cyanobacteria for synthetic biology (Berla et al., 2013).

Strain	Genetic methods	Ideal growth temp (C)	Doubling time (h)	Metabolisms	Genome-scale models?	Notes	References
<i>Synechocystis</i> sp. PCC 6803	Conjugation, natural transformation, Tn5 mutagenesis, fusion PCR	30	6–12	Mixotrophic, autotrophic	Yes	Extensive systems biology datasets are available	Heidorn et al., 2011
<i>Synechococcus elongatus</i> PCC 7942	Conjugation, natural Transformation, Tn5 mutagenesis	38	12–24	Autotrophic	No	A model strain for the study of circadian clocks	Chen et al., 2012
<i>Synechococcus</i> sp. PCC 7002	Conjugation, natural transformation	38	3.5	Mixotrophic, autotrophic	Yes	Among the fastest-growing strains known	Xu et al., 2011
<i>Anabaena variabilis</i> PCC 7120	Conjugation, natural transformation	30	>24	Mixotrophic, autotrophic	No	Nitrogen-fixing, Filamentous	Zhang et al., 2007
<i>Leptolyngbya</i> sp. Strain BL0902	Conjugation, Tn5 mutagenesis	30	~20	Autotrophic	No	Filamentous, Grows well in outdoor photo-bioreactors in a broad range of conditions	Taton et al., 2012

2.4.2 Genetic Engineering

Genetic engineering of cyanobacteria has been focused to divert fixed carbon into metabolic pathways for the production of the desired biofuel or other high-value compound. The first report of exogenous transfer of DNA into cyanobacteria was in 1970 into *Anacystis nidulans* (Shestakov & Khyen, 1970). Since the 1970's numerous advancements have been made in the creation of tools required for genetic engineering and recombinant DNA technologies. Discussions of these genetic engineering technologies of cyanobacteria are heavily prevalent in the literature. The majority of these genetic manipulations were fact finding experiments to better understand genetic, metabolic, and photosynthetic processes of cyanobacteria. However, these experiments and studies were becoming the foundation of later experiments in the creation of cyanobacterial strains for production of biofuels and other high-value compounds. (Elhai, Vepritskiy, Muro-Pastor, Flores, & Wolk, 1997; Golden, Brusslan, & Haselkorn, 1987; Koksharova & Wolk, 2002; Vioque, 2007; Wolk, Vonshak, Kehoe, & Elhai, 1984).

The fundamental concern of the insertion of exogenous DNA into cyanobacteria is the efficiency in which the DNA is transferred to the host. A good example of a naturally competent species is *Synechocystis* sp. PCC 6803 (Barten & Lill, 1995). Naturally competent species of cyanobacteria allow for DNA to pass through the cell membrane with a moderate to high efficiency of DNA uptake. Other species that are known for their ease of genetic transformation include *Synechococcus elongatus* PCC 7942 and *Synechococcus* sp. PCC 7002. Consequentially, the ease of genetic transformation makes these species the most studied for biofuel production. Additional strains of cyanobacteria such as *Anabaena* sp. PCC 7120, *Anabaena variabilis* ATCC 29413, and *Nostoc Punctiforme* ATCC 29133 are popular because of their ability to fix nitrogen. With this additional attribute also comes increased complexity as these species are harder to successfully transform with exogenous DNA. These species contain restriction enzymes that will degrade exogenous DNA that passes through the cell membrane. However, additional transformation techniques have been developed such as conjugation and electroporation which can circumvent these restriction enzymes to allow for genetic manipulation (Bryant, 2006; Wolk et al., 1984). The exogenous DNA that is introduced into the host usually contains a selectable marker. The selectable marker typically encodes for a gene of antibiotic resistance. The selectable marker allows for the selection of positive transformants when cultured on selectable media.

Gene expression is also of great importance for producing a proper genetically engineered strain. To create a viable genetic mutant certain parameters of gene expression need to be addressed such as codon usage, copy number, inducible vs. constitutive gene expression, and promoter activity all need to be optimized in order for

the inserted gene to express properly. The expression of exogenous genes can be performed in two different ways. The first option allows the gene to be incorporated into the host's genome through the use of homologous sequences typically at a "neutral" on the chromosome. The second option allows the gene to be expressed in a self-replicating plasmid or "expression vector" separate from the host's genome. Both of these options has specific advantages and disadvantages. Genes that are incorporated into the host's genome are typically more stable and stay intact in later progeny. However, known "neutral sites" that are chosen because they are located in areas on the chromosome that should not confer detrimental effects can be very limited in some species of cyanobacteria. Some popular species of cyanobacteria that have known "neutral sites" include *Anabaena* sp. PCC 7120, *Synechocystis* sp. PCC 6803, and *Synechococcus* sp. PCC 7942. Expression vectors or self-replicating plasmids allow for the integrity of the host's genome to remain intact. However, due to their nature of self-replicating independent of the genome some inconsistency can occur in the total copy number between cells. To this end many expression vectors are derived from native cyanobacterial plasmids that have uncharacterized plasmid copy numbers. This results in some inconsistency in control and expression of the genes present on the plasmid in cyanobacteria.

2.4.3 Biofuels from Cyanobacteria

Currently, cyanobacteria have been genetically engineered to produce a variety of biofuels and high value compounds. Subsequent sub-sections will discuss various biofuels that have been produced from cyanobacteria.

2.4.3.1 Ethanol

The majority of bioethanol production currently is produced from fermentation processes from agriculture feedstocks. The most notable bioethanol production occurs in Brazil where sugarcane is grown to be converted into bioethanol. Production of bioethanol from cyanobacteria has numerous advantages over traditional agriculture crops. As mentioned previously cyanobacteria has 10-100 fold higher photosynthetic efficiency than traditional plants. The first experiments in studying cyanobacteria to ferment ethanol occurred in 1991. In this study 37 different species of cyanobacteria were analyzed for the fermentation of ethanol. It was found that 16 species were able to ferment and excrete ethanol as the main fermentation product (Heyer & Krumbein, 1991). Typically fermentation is minor pathway in cyanobacteria and only used for the survival of the cell. Genetic engineering of cyanobacteria would be required to produce meaningful amounts of ethanol. The first species of cyanobacteria to be genetically modified to produce ethanol was *Synechococcus* sp. PCC 7942. The production of ethanol was performed by transforming the cyanobacteria with exogenous genes coding for pyruvate decarboxylase and alcohol dehydrogenase II from *Zymomonas mobilis*. Expression of these two genes was driven by a *rbcLS* operon promoter in combination with an *Escherichia coli lac* promoter. The resulting strain obtained a productivity of $(0.002488479 \mu\text{g/mL} \cdot \text{OD}_{730} \text{ unit}^{-1} \cdot \text{day}^{-1})$ (Deng & Coleman, 1999). A more recent study used *Synechocystis* sp. PCC6803 with similar genes and has obtained a production rate of $212 \text{ mg L}^{-1} \text{ day}^{-1}$ (Gao, Zhao, Li, Tan, & Lu, 2012). However, the best production efficiencies have been obtained from the start-up company Joule Unlimited

(<http://www.jouleunlimited.com/>). Joule Unlimited has stated they can produce 25,000 gallons of ethanol per acre per year. Joule Unlimited has stated their target goal would be 25 million gallons per year on their first commercial plant. It has been reported that the production costs have been reduced to \$0.60/gallon with subsidies (\$1.23/gallon without subsidies). It also has been projected that the company is planning on going full commercial by 2017 (Lane, 2015). This production level of ($1 \text{ mg} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$) vastly out-produces any production strain reported in the literature (Devroe et al., 2010).

2.4.3.2 Short Chained Alcohols

There are numerous other biofuels are produced by genetically engineered strains of cyanobacteria. Ethanol is classified as a short chain alcohol but there are additional similar alcohol including isopropanol, isobutanol, 1-butanol, 2-Methyl-1-Butanol. Most of these short chained alcohols are produced natively in minuscule amounts from fermentation processes in other microorganisms. The majority of these short chained alcohol use either pyruvate or acetyl-CoA as their central metabolite (Table 3) (Lai & Lan, 2015).

Table 3: Table of various biofuel targets and species of Cyanobacteria (1) (Lai & Lan, 2015).

Chemical Target	Strain	Promoter(s) Used	Gene(s) Expressed	Gene Knockout(s)	Titer (mg/L)	Days of Cultivation	Relevant Central Metabolite	Comments
Ethanol	PCC 6803	P _{rbc}	<i>pdh</i> , <i>adh2</i>	<i>phb</i>	5500	26	Pyruvate	Two copies of <i>pdh</i> , optimized cultivation
Ethanol	PCC 6803	P _{psbA2}	<i>pdh</i> , <i>adh</i>		550	6	Pyruvate	Decarboxylation of pyruvate serves as efficient driving force
Ethanol	PCC 7942	P _{trc}	<i>pdhP</i> , <i>yqhD</i>		182	10	Acetyl-CoA	Oxygen tolerant aldehyde dehydrogenase
Ethanol	PCC 7942	P _{trc} LS	<i>pdh</i> , <i>adh</i>		0.02	7	pyruvate	expression of <i>pdh</i> and <i>adh</i> on plasmid
Isopropanol	PCC 7942	P _{trc}	<i>thi-atoAD-adiC-adiH</i>		146	15	Acetyl-CoA	Medium optimization, 2 phase cultivation
Isopropanol	PCC 7942	P _{trc}	<i>thi-atoAD-adiC-adiH</i>		26.5	9	Acetyl-CoA	decarboxylation of acetoacetate traps carbon to acetone
1-Butanol	PCC 7942	P _{trc} /P _{LacO1}	<i>ter/nphT7</i> , <i>pdhP</i> , <i>yqhD</i> , <i>crt</i> , <i>hbd</i>		317	12	Acetyl-CoA	Oxygen tolerant aldehyde dehydrogenase
1-Butanol	PCC 7942	P _{trc} /P _{LacO1}	<i>ter/nphT7</i> , <i>bidh</i> , <i>yqhD</i> , <i>crt</i> , <i>hbd</i>		30	17	Acetyl-CoA	ATP driving force through acetoacetyl-CoA synthase
1-Butanol	PCC 7942	P _{trc} /P _{LacO1}	<i>ter/atoB</i> , <i>adhE2</i> , <i>crt</i> , <i>hbd</i>		14.5	7	Acetyl-CoA	Dark anaerobic incubation
Isobutyraldehyde	PCC 7942	P _{trc} /P _{LacO1}	<i>hvd</i> , <i>alcS-ivC-ivD</i>		1100	8	Pyruvate	Decarboxylation of KIV serves as effective driving force. <i>In situ</i> product removal
Isobutanol	PCC 6803	P _{trc}	<i>hvd</i> , <i>adhA</i>		240	21	Pyruvate	Oleyol alcohol trap
Isobutanol	PCC 7942	P _{trc}	<i>hvd</i> , <i>yqhD</i>		450	6	Pyruvate	Decarboxylation of KIV serves as effective driving force. <i>In situ</i> product removal
2-Methylbutanol	PCC 7942	P _{trc}	<i>hvd</i> , <i>yqhD</i> , <i>cimA</i> , <i>ieuBCD</i>		178	10	Pyruvate/ acetyl-CoA	decarboxylation, native highly active AHAS

2.4.3.3 Fatty Acids and Hydrocarbons

Another major category of biofuels include fatty acids and hydrocarbons. Biodiesel is typically derived by chemically reacting short chained alcohols with lipids. Previously, third generation biofuels processed triacylglycerides into biodiesel using transesterification. However, in fourth generation biofuels these fatty acids can be directly produced from cyanobacteria using genetic engineering (Tables 4 and 5)(Lai & Lan, 2015). Hydrocarbons are also natively produced in small amounts by some species of cyanobacteria. Although hydrocarbon biosynthesis is natural in some cyanobacteria it was found that overexpression of these genes has improved productivity for alkane production (Kageyama et al., 2015). It should be noted however that even with these overexpressed genes the productivity of hydrocarbons and fatty acids still remains low which likely indicated by bottlenecks in the metabolic pathways (Choi & Lee, 2013).

Table 4: Table of various biofuel targets and species of Cyanobacteria (2) (Lai & Lan, 2015)

Chemical Target	Strain	Promoter(s) Used	Gene(s) Expressed	Gene Knockout(s)	Titer (mg/L)	Days of Cultivation	Relevant Central Metabolite	Comments
Fatty alcohol	PCC 6803	P _{rbc}	<i>jojoba FAR</i>		0.2	18	Acetyl-CoA	using native fatty-acyl-ACP synthesis & expression of <i>jojoba FAR</i>
Fatty alcohol	PCC 6803	P _{rbc} P _{psbA2}	<i>jojoba FAR</i> <i>nas</i>		0.17	10	Acetyl-CoA	expression of <i>jojoba FAR</i> & overexpression of acyl-ACP synthetase
1,3-Propanediol	PCC 7942	P _{trc}	<i>zADH</i> (<i>C. beijerinckii</i>), <i>yghD</i> , <i>mgsA</i>		150	10	Pyruvate	NADPH utilization
2,3-Butanediol	PCC 6803	P _{trc}	<i>als</i> , <i>aldC</i> , <i>ar</i>		585	28	Pyruvate	Codon optimization
2,3-Butanediol	PCC 7942	PLlacO1	<i>alsS</i> , <i>alsD</i> , <i>adh</i>		2380	20	Pyruvate	pyruvate pool coupled to decarboxylation and product low toxicity
Glycerol	PCC 6803	P _{trc}	<i>gpp2</i>		1068	17	DHAP	Salt stress can stimulate glycerol production even in wildtype for about 0.7 mM
Glycerol	PCC 7942	P _{trc}	<i>gpp1</i>		1170	20	DHAP	aeration, thermodynamically favorable glycerol phosphatase
D-Lactate	PCC 6803	P _{trc}	<i>gidA101</i> , <i>sth</i>		1140	24	Pyruvate	expression of transhydrogenase, codon optimized mutated glycerol dehydrogenase, addition of acetate helped production
D-Lactate	PCC 6803	P _{cpc560}	<i>Dldh</i>	<i>pta</i> , <i>phaCE</i>	1060	4	Pyruvate	knockout of PHB synthesis & acetate formation, expression codon optimized <i>ldh</i> from <i>Lactobacillus delbrueckii</i>
D-Lactate	PCC 7942	P _{trc}	<i>ldhD</i> , <i>lidP</i>		829	10	Pyruvate	expression of lactate transporter, engineered <i>Ldh</i> to use NADPH
D-Lactate	PCC 7942	P _{lac}	<i>ldhA</i> , <i>lidP</i> , <i>udhA</i>		55	4	Pyruvate	Expression of <i>LidP</i> protein from <i>E. coli</i> (transporter)
L-Lactate	PCC 6803	P _{trc}	<i>ldh</i>		1800	40	Pyruvate	Long term production
L-Lactate	PCC 6803	P _{trc2} /P _{trc3}	<i>pkhldh</i>	knockdown <i>PPC</i>	837	14	Pyruvate	codon optimization & natural copy
L-Lactate	PCC 6803	P _{trc} /P _{trc}	<i>ldh</i> , <i>sth</i>		288	14	Pyruvate	expression of transhydrogenase, <i>Bacillus subtilis</i> <i>Ldh</i>
L-Lactate	PCC 6803	P _{psbA2}	<i>ldh</i> , <i>ldhP</i>		15.3	18	Pyruvate	Tested various <i>ldh</i> genes.
3-Hydroxypropionate	PCC 7942	P _{trc}	<i>mcr</i> , <i>mcr</i>		659	16	Acetyl-CoA	selection of best performing malonate semialdehyde reductase (<i>Msr</i>), two NADPH utilizing steps
3-Hydroxypropionate	PCC 7942	P _{trc}	<i>gpp1</i> , <i>dhaB</i> , <i>puuC</i>		31.7	10	DHAP	oxygen sensitive, Dark anaerobic with nutrient limitation

2.4.3.4 Olefins

Olefins are another group of biofuels which are comprised of unsaturated hydrocarbons with one or more double bonds. Some examples of these biofuels include ethylene, isoprene, and terpenoids (Tables 5 and 6) (Lai & Lan, 2015). Ethylene is used as a polymer for the production of polyethylene used in plastics. Ethylene is produced by the enzyme Efe which utilizes α -ketoglutarate to produce ethylene with succinate being produced as a byproduct. This bio-product has been produced from cyanobacteria as early as the 1990s (Fukuda et al., 1994; Sakai, Ogawa, Matsuoka, & Fukuda, 1997). In Recent years, The Dr. Yu group had obtained an ethylene production rate of ($171 \text{ mg} \cdot \text{L}^{-1} \cdot \text{Day}^{-1}$) from *Synechocystis* 6803 (Ungerer et al., 2012). Isoprenoids are produced in cyanobacteria using the methyl-erythritol-4-phosphate (MEP) pathway. Isoprene is

comprised of a five carbon diene which can be used to make latex. Isoprene synthase uses dimethylallyl pyrophosphate (DMAPP) which is part of the MEP pathway to produce isoprene. Isoprene synthase is native enzyme found in plants commonly found in the kudzu vine (Lindberg, Park, & Melis, 2010). Terpenoids are a very large class of bioactive molecules that have great potential as a biofuel. The production of terpenes is involved with the MEP pathway using DMAPP and its isomer isopentenyl diphosphate (IPP) to create a ten carbon metabolite geranyl diphosphate (GPP). Two terpenoids synthases limonene synthase and bisabolene synthase use GPP as the direct precursor in production of limonene (C. Halfmann, L. P. Gu, & R. B. Zhou, 2014) and bisabolene (Chuck Halfmann, 2014)

2.4.3.5 Organic acids

Organic acids are produced organically and have acetic properties. Some examples of organic acids produced from Cyanobacteria include lactate and 3-hydroxybutyrate (Tables 4 and 5). Lactate is used for food preparation and potential use in renewable plastics. Currently lactate is one of the most studied organic acids produced from cyanobacteria. Multiple different avenues are being investigated to improve lactate productivity (Angermayr, Paszota, & Hellingwerf, 2012; Niederholtmeyer, Wolfstadter, Savage, Silver, & Way, 2010). 3-Hydroxyacids are used in the production of polyhydroxyalkanoates for the creation of thermoplastics or elastomeric materials that are both biocompatible and biodegradable. 3-Hydroxyacids are produced from 3-hydroxybutyrate cycle that shares similarities with biosynthesis of butanol and isopropanol (Lai & Lan, 2015).

2.4.3.6 Sugars

Cyanobacteria are typically less tolerant to the presence of chemicals than other heterotrophic microorganisms such as *E. coli*. Some sugars produced by cyanobacteria include sucrose and mannitol (Table 5). Sucrose is an exception in cyanobacteria as this sugar is natively produced as an osmoprotectant. Sucrose is of interest because it is not toxic to cyanobacteria and can be produced for utilization by other heterotroph such as *E. coli*. Mannitol is a sugar used in pharmaceutical and food industries (Lai & Lan, 2015). This sugar has been successfully produced in cyanobacteria using a genetically engineered strain of *Synechococcus* sp. PCC 7002 (Jacobsen & Frigaard, 2014)

2.4.3.7 Diols and Polyol

Diols are a classification of chemical compounds consisting of two hydroxyl groups. Polyol are a class of chemical compounds consisting of multiple hydroxyl groups. Some examples of these compounds produced in cyanobacteria include 2,3-Butanediol, 1,2-Propanediol, and Glycerol (Table 4) (Lai & Lan, 2015). 2,3-Butanediol has applications in plastics and can be used in some pesticides. 2,3-Butanediol has been successfully produced in *Synechocystis* sp. PCC6803 and PCC 6803 (Oliver, Machado, Yoneda, & Atsumi, 2013; P. E. Savakis, Angermayr, & Hellingwerf, 2013). 1,2-Propanediol is used as a chemical feedstock and successfully produced from PCC 7942 (Li & Liao, 2013). Glycerol has been heavily investigated for its uses in producing C3 chemicals such as 1,3-propanediol and 3-hydroxypropionate (Clomburg & Gonzalez, 2013). This bio-product has been photosynthetically produced in both PCC 6803 and PCC 7942 (P. Savakis et al., 2015; Tan, Du, & Lu, 2015).

Table 5: Table of various biofuel targets and species of Cyanobacteria (3) (Lai & Lan, 2015).

Chemical Target	Strain	Promoter(s) Used	Gene(s) Expressed	Gene Knockout(s)	Titer (mg/L)	Days of Cultivation	Relevant Central Metabolite	Comments
3-Hydroxybutyrate	PCC 6803	PtcA/Ptac	<i>te: B.phaA-phaB</i>		533	21	Acetyl-CoA	Nutrient limitation, NADPH
Itaconic acid	PCC 6803	Ptac	<i>cad</i>		14.5	16	Isocitrate	Expression of <i>cad</i>
p-Coumaric acid	PCC 6803	PpsbA2	<i>samS</i>	<i>zir1573</i> (<i>laccase</i>)	82.6	4	Tyrosine	knockout of competing pathway for phenolic compound degradation
Fatty acids	PCC 6803	Ptc/PcpC/Ptc	<i>tesA, fatB1, fatB2/ accBC/accDA, fatB2</i>	<i>aas, pta, phb genes, (see comments)</i>	197	2	Acetyl-CoA	construct six generation strain: extensive knock outs of PHB synthesis, peptidoglycan layer protein, hemolysin-like surface layer protein, cyanophycin synthesis
Fatty acids	PCC 7002	Ptc/PpsbA1	<i>tesA/rbcLS</i>	<i>fadD</i>	131	20	Acetyl-CoA	Overexpression of rubisco
Fatty acids	PCC 7942	Ptc	<i>tesA</i>	<i>aaz</i>	45	20	Acetyl-CoA	knockout of acylACP synthetase blocks utilization of fatty acids
Fatty acids	PCC 7942	Ptc/PpsbA1	<i>fat1/rbcLS</i>	<i>aaz</i>	35	20	Acetyl-CoA	overexpression of ACCase hurts production
D-Mannitol	PCC 7002	PpsbA	<i>mlD, mlp</i>	<i>gigA1, gigA2</i>	1100	12	F6P	codon optimization, artificial carbon sink
Hexose	PCC 7942	Ptc	<i>glt, imvA, galU</i>		45	5	Glc6P	expression of sugar transporter
Sucrose	PCC 7942	Ptc	<i>cscB</i>	<i>imvA, gigC</i>	2700	7	Glc6P	Salt stress, knockout of natural carbon/electron sink
Sucrose	PCC 6803	PpetE	<i>cscB, zps, zpp, ugp</i>	<i>ggpS, ggrCD</i>	140	10	Glc6P	Salt stress, knockout of competing pathways, expression of sucrose synthesis genes
Glucosylglycerol	PCC 6803	--	--	<i>ggrCD, ggpR</i>	981	24	G3P/Glc6P	salt shock, hypoosmotic shock
Ethylene	PCC 6803	Ptc	<i>gfe</i>		240 nL/mL/d		α -Ketoglutarate	compared various promoters, used plasmid based expression
Ethylene	PCC 6803	PpsbA	<i>gfe</i>		171 mg/L/d		α -Ketoglutarate	Multiple copies of EFE
Ethylene	PCC 7942	Ptc	<i>ACS-Ctdoc-ACO-Acdoc-Cip2</i>		81.6 nL/mL/d/OD		SAM	Chimeric protein fusion
Ethylene	PCC 7942	PpsbA1	<i>gfe</i>		10.82 μ L/mL/D/OD		α -Ketoglutarate	choose a strong promoter site in 7942 and rps12-mediated gene replacement
Isoprene	PCC 6803	PpsbA2	<i>IspS (Pueraria montana)</i>		0.35	8	G3P/Pyruvate	Gaseous/aqueous two-phase photobioreactor
Isoprene	PCC 6803	PpsbA2	<i>IspS, hmgS, hmgR, fvi, mk, gmd, pmk</i>		0.3	8	Acetyl-CoA	expression of both pathways to IPP increases isoprene production

Table 6: Table of various biofuel targets and species of Cyanobacteria (4) (Lai & Lan, 2015).

Chemical Target	Strain	Promoter(s) Used	Gene(s) Expressed	Gene Knockout(s)	Titer (mg/L)	Days of Cultivation	Relevant Central Metabolite	Comments
Isoprene	PCC 6803	PpsbA2	<i>IspS (Pueraria montana)</i>		50 μ g/gDCW/d		G3P/Pyruvate	Expression of isoprene synthase
Limone	PCC 6803	Ptc	<i>limS (Schizonepeta tenuifolia), dxs, crtE, ipi</i>		1	30	G3P/Pyruvate	codon optimization, enhancing flux through MEP pathway
Limone	PCC 7002	PcpBA	<i>limS (Mentha spicata)</i>		4	4	G3P/Pyruvate	product trap by dodecane overlay/Lan
Limone	PCC 7120	Pnir-PpsbA1	<i>limS (Picea sitchensis), dxs, ipppp, gpps</i>		0.52	12	G3P/Pyruvate	enhancing flux through MEP pathway by gene overexpression, high light density
Farnesene	PCC 7120	Pnir, PpsbA1	<i>fas (Picea abies)</i>		0.31	15	G3P/Pyruvate	codon optimization
Bisabolene	PCC 7002	PcpBA	<i>bis (Abies grandis)</i>		0.6	4	G3P/Pyruvate	product trap by dodecane overlay
Tocopherols	PCC 6803	PnirA	<i>hpd (Arabidopsis thaliana)</i>		0.250 mg/gDCW	12	G3P/Pyruvate	Nitrate inducible promoter
β -Caryophyllene	PCC 6803	PpsbA2	<i>QHS1</i>		0.046	7	G3P/Pyruvate	use similar pathway in 6803 to produce plant's second metabolite, only need few key enzyme
β -Phellandrene	PCC 6803	PpsbA2-trc-T7	<i>PLHS (Lavandula angustifolia)</i>		0.9	2	G3P/Pyruvate	codon optimization, High light with ppsbA2-trc-T7 fused promoter
β -Phellandrene	PCC 6803	PpsbA2	<i>PHLS (Lavandula angustifolia)</i>		0.2	8	G3P/Pyruvate	codon optimization
Dihydroxyacetone	PCC 7942	Ptc	<i>gpp1, dhaD</i>		78.6	16	DHAP	NAD-dependent DhaD could not efficiently reduce glycerol
Acetone	PCC 6803	Ptc/PcpC	<i>cfAB/adc</i>	<i>phaCE, pta</i>	36	4	Acetyl-CoA	Increasing acetyl-CoA pool
Alkanes	PCC 7120	Pado	<i>aar, ado (A. halophytica)</i>		1.25 mg/gDCW	5	Acetyl-CoA	Salt stress

2.4.4 Metabolic profiles

Metabolic profiling of the carbon flux inside cyanobacteria is an important step in determining ways to improve biofuel production. Simply adding genes that are necessary for the production of a particular biofuel or high-value compound is usually not enough to produce these products in meaningful amounts for later commercialization. Commonly these metabolic profiles are characterized through the use of isotope tracers. Coupling characterized metabolic profiles with computational systems biology technologies allows for researchers to create new avenues for metabolic engineering. A prime example of extensive metabolic profiling has been conducted on *Synechosystis* sp. PCC 6803. Investigation of the metabolic systems in this species has allowed for detailed reconstruction of enzyme-catalyzed reactions involved in cellular growth and maintenance (Knoop et al., 2013; Knoop, Zilliges, Lockau, & Steuer, 2010).

2.4.5 Metabolic Engineering

After metabolic cycles have been characterized in a cyanobacterial species, the second step is the improvement of the metabolic cycle to allow for higher productivities of the biofuel. Metabolic engineering aims to direct the carbon flux of the organism to the metabolic pathway that produces the desired product or increase photosynthetic efficiency. Metabolic engineering can accomplish this through variety of ways such as introducing additional genes that increase carbon flux into the desired metabolic pathway. One important avenue for increasing carbon flux to the desired metabolic pathway is the use of autotrophic carbon fixation pathways.

All life on earth is carbon based which makes autotrophic carbon fixation the most crucial biosynthetic process in the biosphere. Carbon fixation is responsible for the net fixation of 7×10^{16} grams of carbon annually, which results in the conservation of 2.8×10^{18} kJ of energy annually (Geitmann, 2005). Autotrophic carbon fixation cycles have been studied with great interest to increase carbon fixation in biofuel producing strains of cyanobacteria. This section will discuss the major known autotrophic CO₂ carbon fixation cycles that exist in nature that may allow for engineering an increased carbon fixation.

2.4.5.1 Calvin-Benson Reductive Pentose Phosphate Cycle

The Reductive Pentose Phosphate Cycle or Calvin-Benson cycle is the most important carbon fixation pathway in nature. RubisCO is responsible for the catalysis of the electrophilic addition of CO₂ to the C₅ sugar ribulose-1,5-bisphosphate. (Figure 8) (I. A. Berg, 2011). The CB cycle can operate in some thermophiles but never hyperthermophiles as the upper temperature limit is 70-75°C (Imanaka, Fukui, Atomi, & Imanaka, 2002; Phillips & Thornalley, 1993). The most crucial enzyme in this cycle is RubisCO which is the most abundant protein in the biosphere (Ellis, 1979). However, RubisCO is notoriously catalytically inefficient with a low affinity for CO₂ and low catalytic turnover rate. This low affinity for CO₂ leads to a squandering oxygenase reaction that is responsible for photorespiration (Kachru & Anderson, 1974). Photorespiration is likely an inevitable event in an oxygen rich atmosphere because of the lack of discrimination between CO₂ and O₂ in RubisCO (Lorimer & Andrews, 1973). It has been determined that the majority of the isoenzymes of RubisCO have been optimized for their environment. This depressingly creates a situation in which an

optimized enzyme of RubisCO for maximum velocity would simultaneously decrease affinity for the substrates (Tcherkez, Farquhar, & Andrews, 2006).

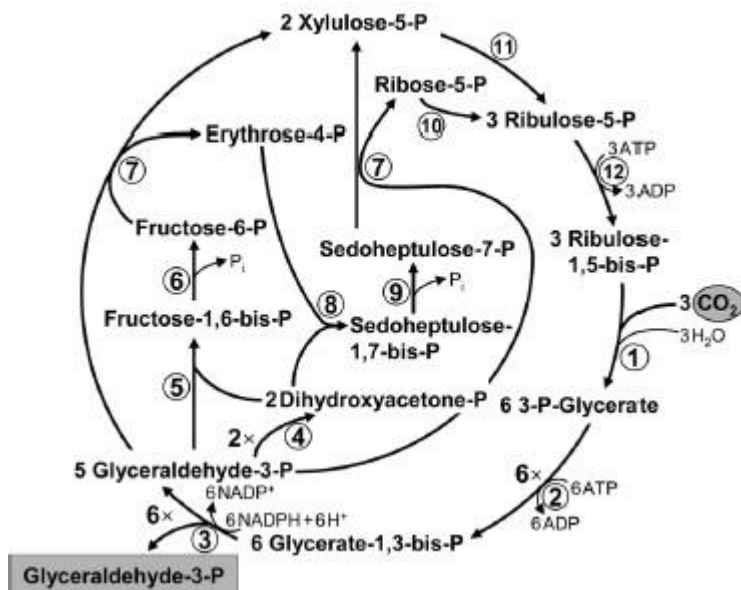


Figure 8: The reductive pentose phosphate (Calvin-Benson) cycle. Enzymes: 1) ribulose-1,5-bisphosphate carboxylase/oxygenase; 2) 3-phosphoglycerate kinase; 3) glyceraldehyde-3-phosphate dehydrogenase; 4) triose-phosphate isomerase; 5) fructose-bisphosphate aldolase; 6) fructose-bisphosphate phosphatase; 7) transketolase; 8) sedoheptulose-bisphosphate aldolase; 9) sedoheptulose-bisphosphate phosphatase; 10) ribose-phosphate isomerase; 11) ribulose-phosphate epimerase; and 12) phosphoribulokinase.

2.4.5.2 The Reductive Citric Acid Cycle

The Reductive Citric Acid Cycle or more commonly known as the reductive tricarboxylic acid cycle (rTCA) was first identified in green sulfur bacterium *Chlorobium limicola* (Evans, Buchanan, & Arnon, 1966; G Fuchs, 1989; Georg Fuchs & Stupperich, 1978). It is believed to primarily exist in anaerobic and microaerobic organisms but has not presently been found in the archaeal domain (Ivan A Berg et al., 2010). The rTCA cycle reverses the reactions found in the oxidative citric acid cycle or also known as the Krebs cycle. The rTCA cycle utilizes two molecules of CO₂ to produce acetyl-CoA

(Figure 9) (I. A. Berg, 2011). Acetyl-CoA must be converted into other central intermediates in the carbon metabolism. For example, pyruvate/phosphoenolpyruvate (PEP) must be converted to oxaloacetate/2-oxoglutarate. This results in the requirement for additional enzymes for proper acetyl-CoA assimilation. Acetyl-CoA can be reductively carboxylated into pyruvate by ferredoxin-dependent pyruvate synthase. Pyruvate can later be converted into PEP (I. A. Berg, 2011; Evans et al., 1966; Shiba, Kawasumi, Igarashi, Kodama, & Minoda, 1985). The rTCA cycle has the capability to use both ferredoxin and NAD(P)H as electron donors (Miura, Kameya, Arai, Ishii, & Igarashi, 2008). It should be noted that this carbon fixation cycle only requires the use of two ATP equivalents to form pyruvate and three additional ATPs to convert to triosephosphates. This is a significant advantage over the CB cycle as it is less energy consuming to net the same amount of pyruvate. The lower energy cost is mainly attributed to the FeS photosynthetic reaction center type I capable of direct reduction of ferredoxin (I. A. Berg, 2011; Bryant & Frigaard, 2006). Although this carbon fixation cycle is much more energy efficient its sensitivity to O₂ restricts the applications of introducing this pathway into other hosts.

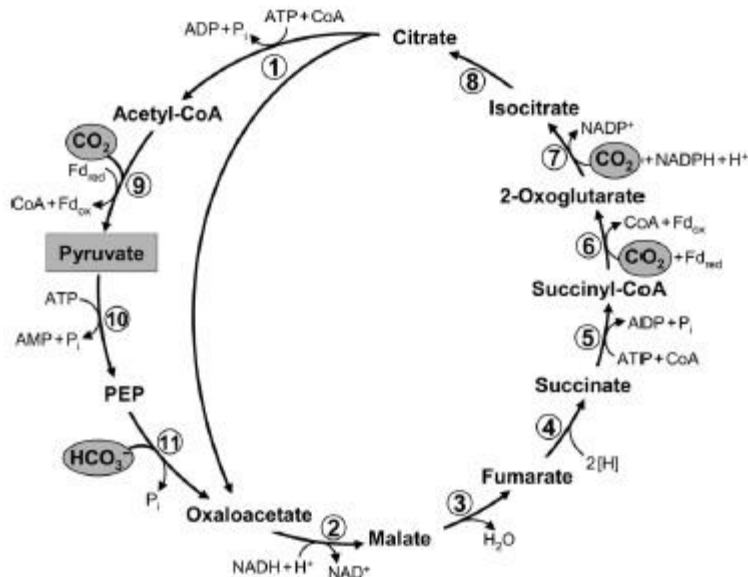


Figure 9: The reductive citric acid (rTCA) cycle as it functions in green sulfur bacteria (Evans et al., 1966; Ivanovsky, Sintsov, & Kondratieva, 1980). The pathway of acetyl-CoA assimilation to pyruvate, phosphoenolpyruvate (PEP), and oxaloacetate is shown as well. Enzymes as follows: 1) ATP-citrate lyase; 2) malate dehydrogenase; 3) fumarate hydratase; 4) fumarate reductase (natural electron donor is not known); 5) succinyl-CoA synthetase; 6) ferredoxin (Fd)-dependent 2-oxoglutarate synthase; 7) isocitrate dehydrogenase; 8) aconitate hydratase; 9) Fd-dependent pyruvate synthase; 10) PEP synthase; 11) PEP carboxylase. (adapted from (I. A. Berg, 2011)).

2.4.5.3 The Reductive Acetyl-CoA Pathway

The Reductive Acetyl-CoA pathway is an exotic non-cyclical carbon fixation cycle. This pathway allows for the conversion of two CO_2 molecules to form acetyl-CoA. This is accomplished through the use of coenzyme and an enzyme metal reaction center as the CO_2 acceptors (Figure 10) (I. A. Berg, 2011; Ljungdhal, 1986). The mechanics behind the carbon fixation starts with one molecule of CO_2 which is reduced to a methyl group and bound to a tetrahydropterin coenzyme. The second CO_2 molecule is then reduced to carbon monoxide which is bound to a nickel molecule located in the reaction center of CO dehydrogenase. The CO dehydrogenase performs as an acetyl-CoA

synthase in this pathway. This enzyme then accepts the methyl group from the methylated tetrahydropterin which is aided by a methylated corrinoid protein. These reactions are then combined to form an enzyme bound nickel acetyl group complex and released by coenzyme A to create acetyl-CoA. As typical for carbon fixation cycles the enzymes responsible for carbon fixation in this pathway may contribute to 6%-9% of the total soluble protein present in these organisms (I. A. Berg, 2011; Roberts, Lu, & Ragsdale, 1992). This pathway is almost primarily used by methanogens and acetogens for carbon fixation and energy conservation during autotrophic growth. It should be noted that there exist a large amount of variants of this cycle in both coenzymes and electron carriers. Interestingly, the pathway functions without the input of ATP in methanogens and instead uses chemiosmotical energy for the reduction of ferredoxin and for the reduction of CO_2 into formylmethanofuran. However, this carbon fixation cycle requires strict anoxic environment in order to function properly as one of the central enzymes CO dehydrogenase is highly oxygen sensitive (I. A. Berg, 2011).

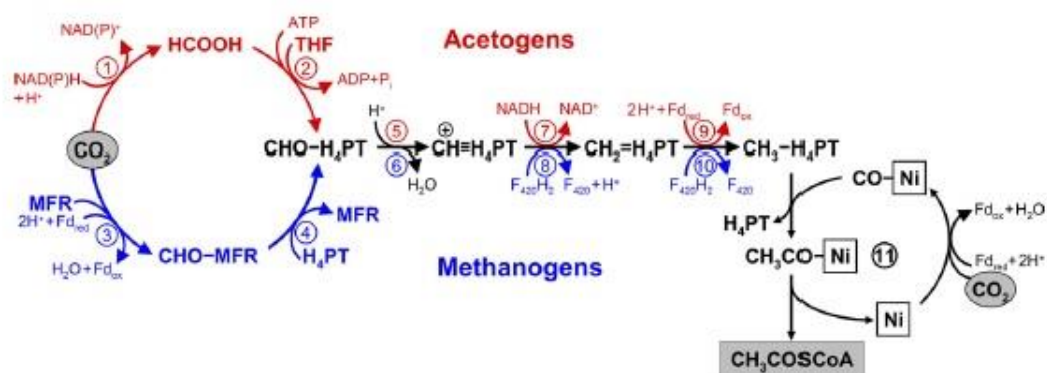


Figure 10: The reductive acetyl-CoA pathway. One CO_2 molecule is reduced to carbon monoxide which is bound to a nickel atom in the reaction center of CO dehydrogenase, and another CO_2 molecule is reduced to a methyl group bound to the carrier protein tetrahydropterin; next the methyl group is transferred to nickel-bound CO results in acetyl-

CoA synthesis. The upper part (red) shows the variant of the pathway as it functions in acetogens, and the lower part (blue) depicts the pathway as it functions in methanogens. Enzymes: 1) formate dehydrogenase; 2) formyl-THF synthetase; 3) formyl-MFR dehydrogenase; 4) formyl-MFR:tetrahydromethanopterin formyltransferase; 5) methenyl-THF cyclohydrolase; 6) methenyl-tetrahydromethanopterin cyclohydrolase; 7) methylene-THF dehydrogenase; 8) methylene-tetrahydromethanopterin dehydrogenase; 9) methylene-THF reductase; 10) methylene-tetrahydromethanopterin reductase; 11) CO dehydrogenase/acetyl-CoA synthase. (adapted from (I. A. Berg, 2011)).

2.4.5.4 3-Hydroxypropionate Cycles

There are two main types of 3-hydroxypropionate cycles (3-HPA): 3-hydroxypropionate bi-cycle and the 3-hydroxypropionate cycle. The 3-hydroxypropionate bi-cycle was first discovered in *Chloroflexus aurantiacus* by Helge Holo and later fully characterized by Georg Fuchs (Herter, Fuchs, Bacher, & Eisenreich, 2002; Holo, 1989). It was originally believed that glyoxylate was the primary product produced from the carbon fixation cycle. However, later studies concluded that glyoxylate was not the primary product and another pathway was found that used glyoxylate for cellular metabolic building blocks thus creating the fully characterized bi-cycle (Herter et al., 2002). The 3-hydroxypropionate bi-cycle begins with glyoxylate synthesis in which acetyl-CoA is carboxylated into malonyl-CoA. Malonyl-CoA is then reduced by 3-hydroxypropionate into propionyl-CoA. Propionyl-CoA is then carboxylated and a carbon rearrangement occurs that yields succinyl-CoA. Afterwards succinyl-CoA is converted into (S)-methylmalonyl-CoA which is then cleaved into acetyl-CoA and glyoxylate that regenerates the precursor molecule acetyl-CoA. The second part of the bi-cycle then utilizes the freed glyoxylate to combine with propionyl-CoA to form β -methylmalonyl-CoA. β -methylmalonyl-CoA is then combined with mesaconyl-CoA and converted into citramalonyl-CoA. The resulting citramalonyl-CoA is then cleaved into

pyruvate and acetyl-CoA. The recently cleaved acetyl-CoA is then converted to propionyl-CoA which is used to regenerate the cycle again (Herter et al., 2002). It should be noted that the only autotroph that utilizes the 3-hydroxypropionate bicycle is *Chloroflexus aurantiacus*. It should be noted that this oxygen tolerant 3-hydroxypropionate bi-cycle is energy intensive requiring seven ATP for the synthesis of pyruvate but the biotin-dependent acetyl-CoA/propionyl-CoA carboxylase(s) are irreversible and uses bicarbonate as an active inorganic carbon species (I. A. Berg, 2011). It is interesting to note that this cycle has multiple bi- and multi-functional enzymes with a total of 19 steps but only 13 enzymes are required and is completely functional in the presence of oxygen (Figure 11) (Zarzycki, Brecht, Müller, & Fuchs, 2009).

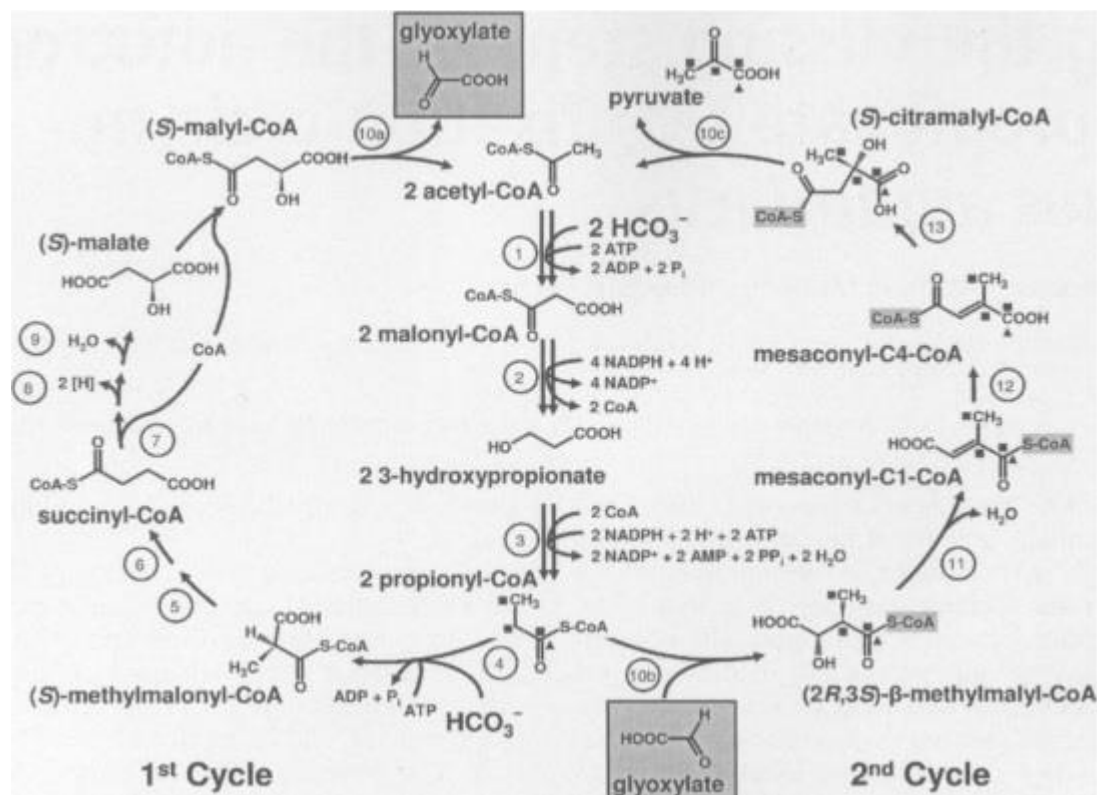


Figure 11: The complete 3-hydroxypropionate bi-cycle, as studied in *C. aurantiacus*. 1) Acetyl-CoA carboxylase, 2) malonyl-CoA reductase, 3) propionyl-CoA synthase, 4) propionyl-CoA carboxylase, 5) methylmalonyl-CoA epimerase, 6) methylmalonyl-CoA mutase, 7) succinyl-CoA:(S)-malate-CoA transferase, 8) succinate dehydrogenase, 9)

fumarate hydratase, 10)(a, b, c) (S)-malylyl-CoA/ β -methylmalylyl-CoA/(S)-citramalylyl-CoA (MMC) lyase, 11) mesaconyl-C1-CoA hydratase (β -methylmalylyl-CoA dehydratase), 12) mesaconyl-CoA C1-C4 CoA transferase, 13) mesaconyl-C4-CoA hydratase. (Adapted from (Zarzycki et al., 2009)).

A very similar pathway to 3-hydroxypropionate bi-cycle is the 3-hydroxypropionate cycle. This cycle is only one half of the previously stated bi-cycle in which the glyoxylate is used as the precursor molecule and produces pyruvate. This cycle should be self-sustaining if the pathway is introduced in an organism that already utilizes the Calvin-Benson cycle (Figure 12). Since this cycle is just a portion of the 3-HPA bicycle refer to the previously discussed pathway in the 3-HPA bi-cycle as this cycle is just one half of the bi-cycle. This cycle was designed to act as a photorespiratory bypass as well as an additional carbon fixation cycle that would supplement the already present CB cycle. All the enzymatic reactions in this cycle are oxygen tolerant and first introduced into *Synechococcus elongatus* PCC7942 (Shih, Zarzycki, Niyogi, & Kerfeld, 2014). This pathway if integrated properly into the cyanobacteria should avoid the net loss of nitrogen and carbon in the photorespiratory C2 cycle and should result in a net gain in carbon fixation due to enzyme acetyl-CoA carboxylase (ACCase). This pathway could substantially increase production of pyruvate in the organism thus allowing for carbon to be used in downstream metabolic processes for ethanol production in genetically engineered strains of cyanobacteria (Shih et al., 2014). Although in the study that explored the addition of this pathway did not see significant growth increase it could be functional in other species of cyanobacteria.

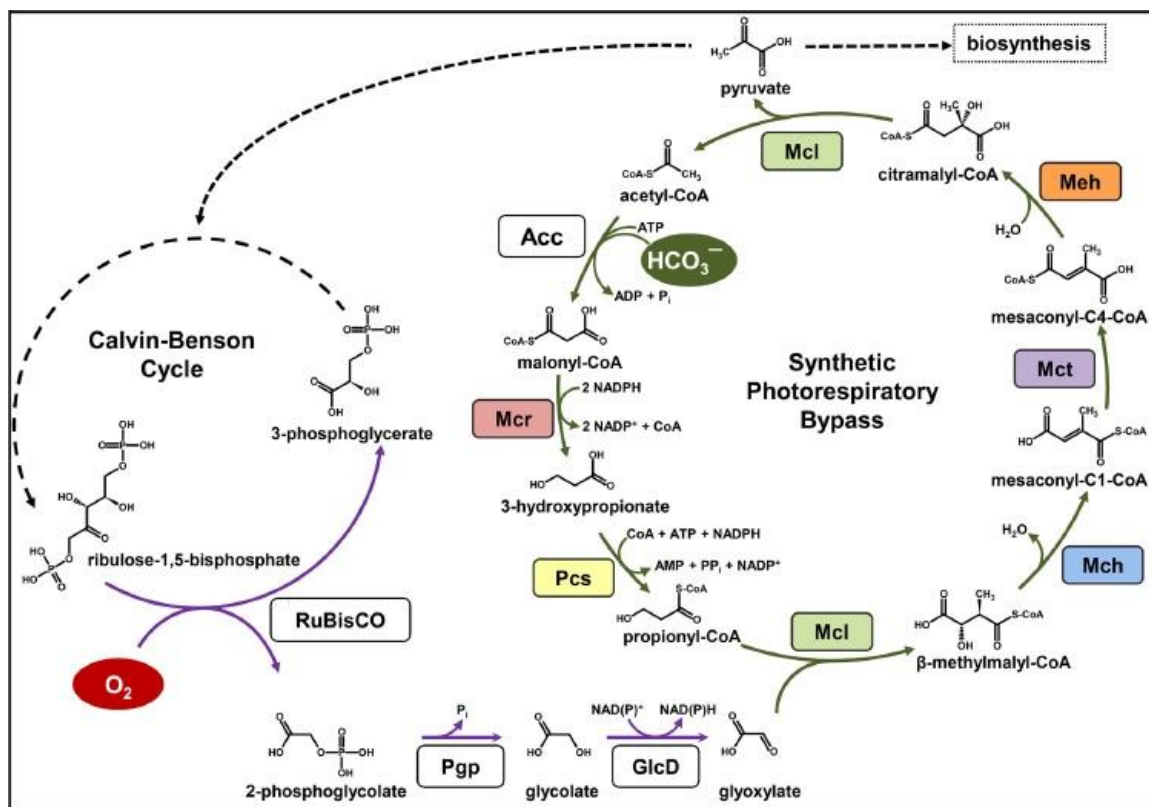


Figure 12: Engineered synthetic photorespiratory bypass based on part of the 3-HPA bi-cycle, which also fixes bicarbonate. An overview of the designed pathway and its intersection with the CB cycle is visualized. Enzymes in white boxes are commonly present in cyanobacteria and plants. The 6 additional enzymes required to create this CO_2 fixing photorespiratory bypass are in colored boxes. One bicarbonate molecule is fixed, while one glyoxylate is consumed to form pyruvate, which can be used for biosynthesis or to replenish the CB cycle. Abbreviations: (CB), Calvin-Benson; (RuBisCO), ribulose-1,5-bisphosphate carboxylase/oxygenase; (3-HPA), 3-hydroxypropionate; (IPTG), isopropyl-1-thio- β -D-galactopyranoside; (MCH), mesaconyl-C1-CoA hydratase; (MCT), mesaconyl-CoA C1:C4 CoA transferase; (MCL), malyl-CoA lyase; (MEH), mesaconyl-C4-CoA hydratase; (MCR), malonyl-CoA reductase; (PCS), propionyl-CoA synthase; (ACCase), acetyl-CoA carboxylase. (adapted from (Shih et al., 2014)).

Chapter 3: Genetic Engineering of *E. coli* W for the Production of Ethanol from Beet Juice Concentrate

ABSTRACT

The aim of this study was to engineer a sucrose-utilizing *E. coli* W strain to produce bioethanol using the beet juice as the sole carbon source. Genes coding for pyruvate decarboxylase (*pdh*) and alcohol dehydrogenase 2 (*adhB*) from *Zymomonas mobilis* were sub-cloned into pZR2011. The pZR2011 was then transformed into *E. coli* W for testing production of ethanol using beet juice. The beet juice concentrate was diluted using BG-11 media into 1:20 and 1:30 dilutions which equaled 3% and 2% sucrose by volume. The *E. coli* W bearing pZR2011 produced substantial amount of ethanol using both dilutions of beet juice. Ethanol production and subsequent sucrose consumption in the growth media concludes that the engineered *E. coli* W is capable of producing and secreting ethanol using only diluted beet juice and mineralized water (BG-11 medium).

3.1 Introduction

One of the biggest challenges that humanity faces today is meeting the growing global energy demand. Reliance on fossil fuels must be gradually phased out and more reliance should be placed on renewable resources. Currently in the U.S. most of the biofuel produced from corn starch is economically viable with only government subsidies and high oil prices (United Nations Conference on, Development, & Larson, 2008).

Industrial beets (*Beta vulgaris* L.) are becoming an ideal feedstock for current biorefinery. Beets produce readily-fermentable sucrose, eliminating the costs for pretreatment and saccharification required for traditional starch/cellulose biorefinery feedstocks. Moreover, industrial beets had a 1.7-fold higher productivity of sugar in hexose equivalents (12.6 Mg ha⁻¹) than that of corn (NDSU CREC, 2014; USDA-ERS, 2014). However, the whole beet has very high moisture content (75% water), which is a big challenge for transportation and storage. Thus, the success of industrial beets as an ideal feedstock depends largely on the development of energy-efficient systems for sugar storage and subsequent on-site conversion to biofuel. Researchers have successfully developed a process to recover and store sucrose from industrial beets, which allows year-round processing (Vargas-Ramirez et al., 2013). One of the most advantageous properties of using sugar beets instead of starch based feedstocks is due to the lower processing costs. Bioconversion of corn into bioethanol requires the feedstock to be hydrolyzed in order to produce the fermentable sugars required for downstream conversion into bioethanol (Yan Lin, 2006). Sugar beets are almost entirely composed of sucrose as the primary sugar which breaks down into the simple sugars fructose and

glucose (Kenneth A. Leiper, 2006). To produce ethanol from sugar beets year round storage infrastructure will be required; recently a method of storage which turned the sugar beets into a raw thick juice using a film evaporator was discovered that could preserve 99% of the fermentable sugars for at least 24 weeks (Vargas-Ramirez, Haagenson, Pryor, & Wiesenborn, 2013). This study used raw thick beet juice to produce ethanol using genetically modified *E. coli* W (ATCC9637 referred as *E. coli* W). *E. coli* W was chosen to produce ethanol because of its ability to grow well using both sucrose and glucose carbon sources (Arifin et al., 2014). The utilization of sucrose by *E. coli* W is because of the presence of *csc* regulon which encodes for: a regulator (*cscR*), a sucrose transporter (*cscB*), an invertase (*cscA*); and a fructokinase (*cscK*) (Akdağ & Çalık, 2015). Additional properties were considered when choosing *E. coli* W as the production vessel. It has been shown that there is lower net acetate produced in *E. coli* W. High acetate production in other *E. coli* strains can limit the maximum amount of sucrose conversion to fuel ethanol and also cause lower production titers. In this study, *E. coli* W was genetically modified by transforming with plasmid pZR2011 containing genes coding for pyruvate decarboxylase (*pdh*) and alcohol dehydrogenase 2 (*adhB*), allowing for the organism to uptake sucrose and convert it into bioethanol via glycolysis – pyruvate – acetaldehyde – ethanol.

3.2 Materials and Methods

3.2.1 Construction of pZR2011

pZR2011 (Table 8) was an ethanol producing plasmid created from pZR672 (Table 8) and used pZR1188 (Table 8) as the backbone. The DNA fragment containing

the ethanol producing genes (*pdh*, *adhB*) from pZR672 was digested using *Xba*I & *Bam*HI, ligated into *Avr*II & *Bam*HI digested pZR1188 vector. After confirmed by colony PCR using primers ZR45 & ZR453 (Table 7), the resulting plasmid designated pZR2011 was further transformed into *E. coli* W creating ethanol producing strain designated S4486.

Table 7: Primer used in the verification of pZR2011

Primer name	Oligonucleotide sequence (5' → 3')*	Purpose
ZR45	CCTCGTAGAACTAGCAAAG	Used for verification of pZR2011, genes (<i>pdh</i> , <i>adhB</i>)
ZR453	TCTCGAGCTTGTCATCGTCATCCTTGTAATC	

Table 8: List of Strains and plasmids used in this study

Plasmid or strain	Relevant characteristic(s)**	Source or reference
Plasmids		
pZR1188	Shuttle Vector; Nm ^r /Km ^r	(C. Halfmann et al., 2014)
pZR672	Shuttle vector; Ethanol producing plasmid containing 2 ethanol production genes (<i>pdh</i> , <i>adhB</i>); Nm ^r /Km ^r	(Zhou R., 2009)
pZR2011	Shuttle vector; Ethanol producing plasmid containing 2 ethanol production genes (<i>pdh</i> , <i>adhB</i>); Nm ^r /Km ^r	This Study
Bacterial Strains		
NEB10β	Cloning host	New England Biolabs
<i>E. coli</i> W (ATCC 9637)	<i>E. coli</i> Host for genetic engineering to utilize sucrose from beet juice and produce ethanol	(Arifin et al., 2014)
S4486	<i>E. coli</i> W mutant strain harboring plasmid to overexpress <i>pdh</i> and <i>adhB</i>	This study

^{**} Ap ^r , ampicillin resistance; Sp ^r /Sm ^r , spectinomycin-streptomycin resistance; Tc ^r , tetracycline resistance; Nm ^r /Km ^r , neomycin-kanamycin resistance; Cm ^r /Em ^r chloramphenicol-erythromycin resistance		

3.2.2 Preparation of Beet Juice Cultures

Beet juice concentrate was prepared into a stock solution by creating a 1:10 dilution using 35mL of Beet juice concentrate and 315mL of BG-11. The stock solution was then pasteurized by heating the solution to 100°C for 10 minutes. Stock solution was allowed to cool to room temperature over the period of 2 hours. Stock solution was then used to create 50 mL volume experiment cultures at 1:20 and 1:30 dilutions using BG-11. All experiment cultures contained 50 µg/mL of kanamycin and 20 mM of NH₄Cl.

3.2.3 Ethanol production from Beet juice experiment

The day before the start of the experiment S4486 (*E. coli* W bearing pZR2011) was inoculated into 5 mL of LB and 50 µg/mL of kanamycin was added. The culture was incubated at 37°C and 250 RPM. Before the start of the experiment overnight culture was washed 3 times by centrifugation at 12,000 g for 1 min and then resuspended in BG-11 media. Final wash was resuspended in 1mL of BG-11 as seed culture. All experiment cultures were inoculated with 200 µL of washed seed culture above. Schedule of samples taken were conducted as listed in Table 9. O.D. readings were performed using 30µL of culture aliquot into 270 µL of BG-11 (dilution of 10 times) in Costar 96 (Corning™ mfr. No. 3615) well plate and O.D. readings were taken at 600 nm using the Plate Reader (BioTek Synergy 2 Multi-detection Microplate Reader). One milliliter of media was also taken at every time point and placed into a 1.5mL eppendorf tube for

HPLC analysis. Culture was refreshed by adding 1mL of stock culture that was inoculated in the exact same conditions as experiment cultures to maintain proper volume of the cultures. Media samples were then centrifuged at 12,000 g for 10 minutes. Supernatant was then removed and placed into a fresh 1.5mL eppendorf tube and frozen overnight at -20°C. After freezing the samples were then thawed at room temperature and then passed through 0.20 µm filter and placed into a HPLC vial for HPLC analysis. Samples were taken as described in Table 9.

Table 9: Time schedule for media samples and O.D._{600nm} readings.

Day 0		
Time	Hour	Sample
8:00 AM	0	1
11:00 AM	3	2
2:00 PM	6	3
5:00 PM	9	4
8:00 PM	12	5
11:00 PM	15	6
Day 1		
2:00 AM	18	7
5:00 AM	21	8
8:00 AM	24	9
2:00PM	30	10
8:00 PM	36	11
Day 2		
2:00 AM	42	12
8:00 AM	48	13

3.2.4 HPLC Analysis

An Agilent HPLC (1220 Infinity II LC System) with a refractive index detector was used. Reaction products and standard compounds were detected by absorbance at 260 nm. A RHM-monosacchride H⁺ column was used (Rezex 7.8mm × 250mm, Rezex) was equilibrated at a flow rate of 0.6 ml min⁻¹ with ddH₂O water. An isocratic program

with a runtime of 25 min was used. Ethanol concentration was identified through the use of an ethanol standard curve and a retention time of 22 minutes. Sucrose concentration was identified using a sucrose standard curve and a retention time of 9.1 minutes.

3.3 Results

3.3.1 Verification of positive mutants bearing pZR2011

Verification of positive mutants bearing pZR2011 was performed on single colonies of *E.coli* W on antibiotic resistant plate using primers ZR45 and ZR453 (Figure 13).

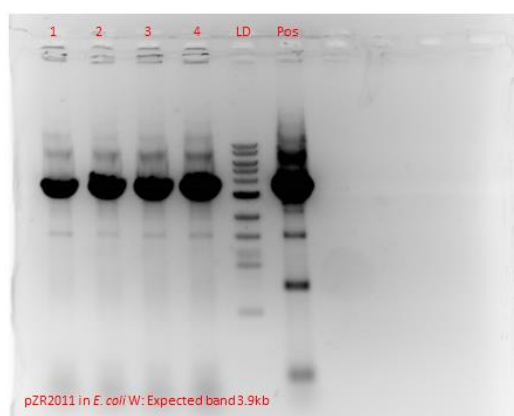


Figure 13: Colony PCR of colonies of *E.coli* W. Colonies were positive for the 3.9 kb band (genes *adhB* and *pdh*). Colonies 1-4 were all positive for the insert. Positive control and ladder are listed as (Pos) and (LD), respectively.

3.3.2 Growth Experiment

Two cultures were prepared using the best two dilutions of beet juice concentrate 1:20 and 1:30 as they showed the highest amount of growth among the tested dilutions (data not shown). Growth data (Figure 14) revealed that 1:20 dilution has the best amount of growth in 54 hours. 1:30 dilution also performed similarly but growth was slightly slower.

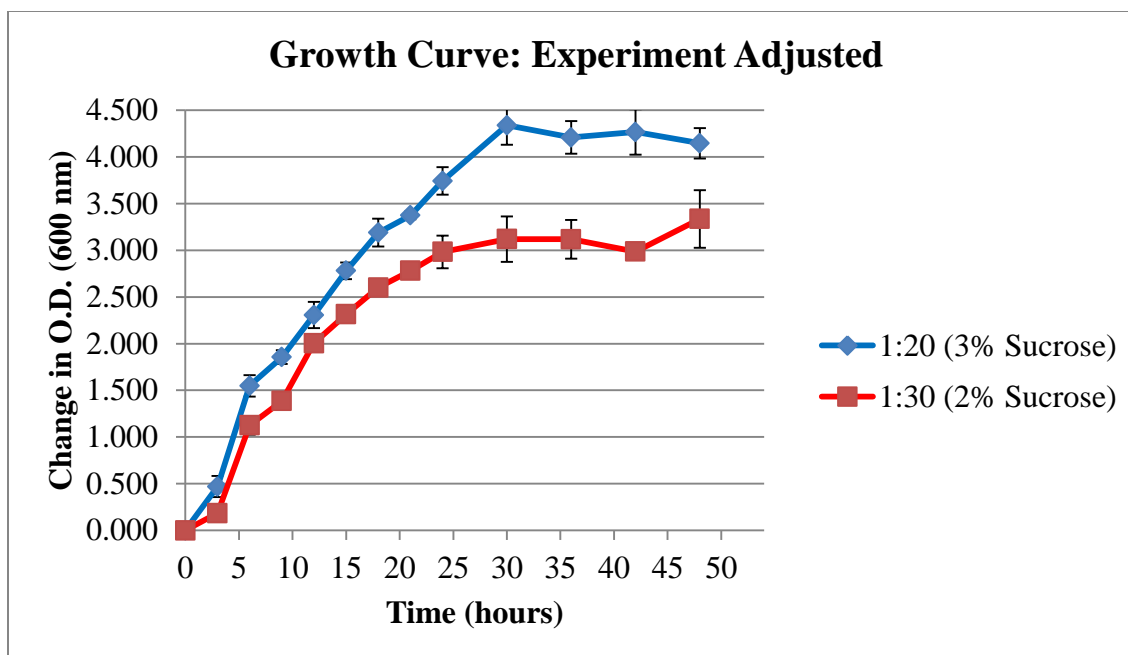


Figure 14: Growth experiment for 1:20 and 1:30 dilutions of beet juice concentrate. Cultures of *E. coli* W bearing the pZR2011 plasmid in 1:20 and 1:30 diluted beet juice concentrate with BG-11.

3.3.3 Ethanol Production

Ethanol production was monitored during the experiment by removing 1mL of growth media and then preparing it for HPLC analysis (Figure 15). It was observed that the fastest growing dilution of beet juice concentrate (1:20) also produced the most amount of ethanol. 1:30 dilution behaved similarly but was slightly lower production of ethanol. Ethanol production rate was also calculated from the ethanol concentration data (Figure 16). Ethanol production rate revealed that the 1:30 dilution of beet juice concentrate had the highest ethanol production rate (18.8 mg/L/H/OD₆₀₀ at hour 3) when compared to the 1:20 dilution (12.5 mg/L/H/OD₆₀₀ at hour 3).

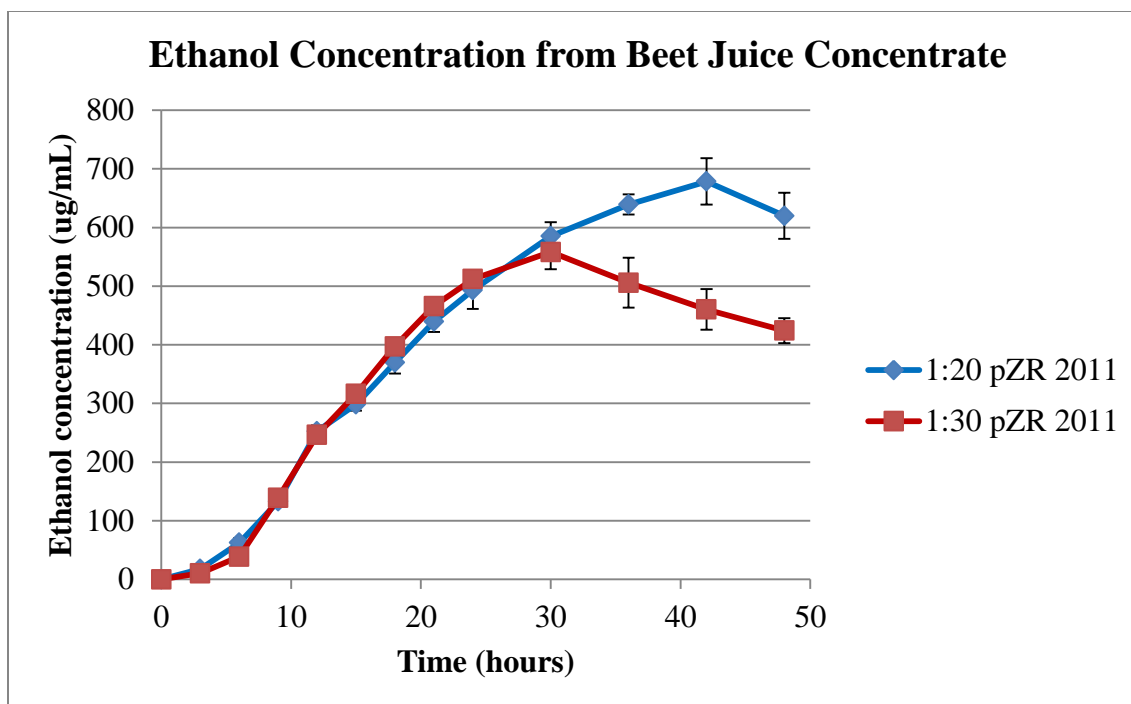


Figure 15: Ethanol Production from Beet Juice Concentrate. Ethanol concentration from *E. coli* W bearing pZR2011 for 48 hours. Data shows the amount of ethanol present in the media at each time point. Both dilutions of beet juice concentrate that were cultured contained pZR2011.

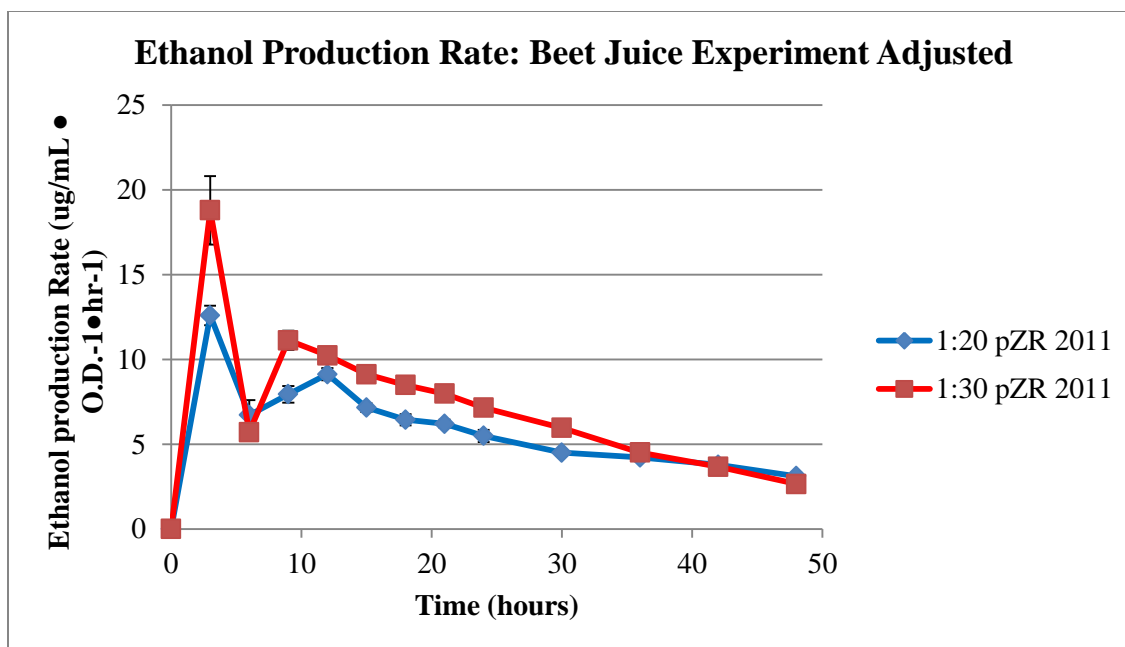


Figure 16: Ethanol Production Rate. Ethanol production rate from *E. coli* W bearing pZR2011 for a duration of 48 hours.

3.3.4 Sucrose consumption

Sucrose concentration in growth media was also monitored using HPLC analysis for the two different beet juice concentrate dilutions (Figure 17). The sucrose showed decreasing concentration over the span of 48 hours. 1:20 dilution had the highest starting concentration as expected. 1:30 dilution showed very similar consumption at different time points.

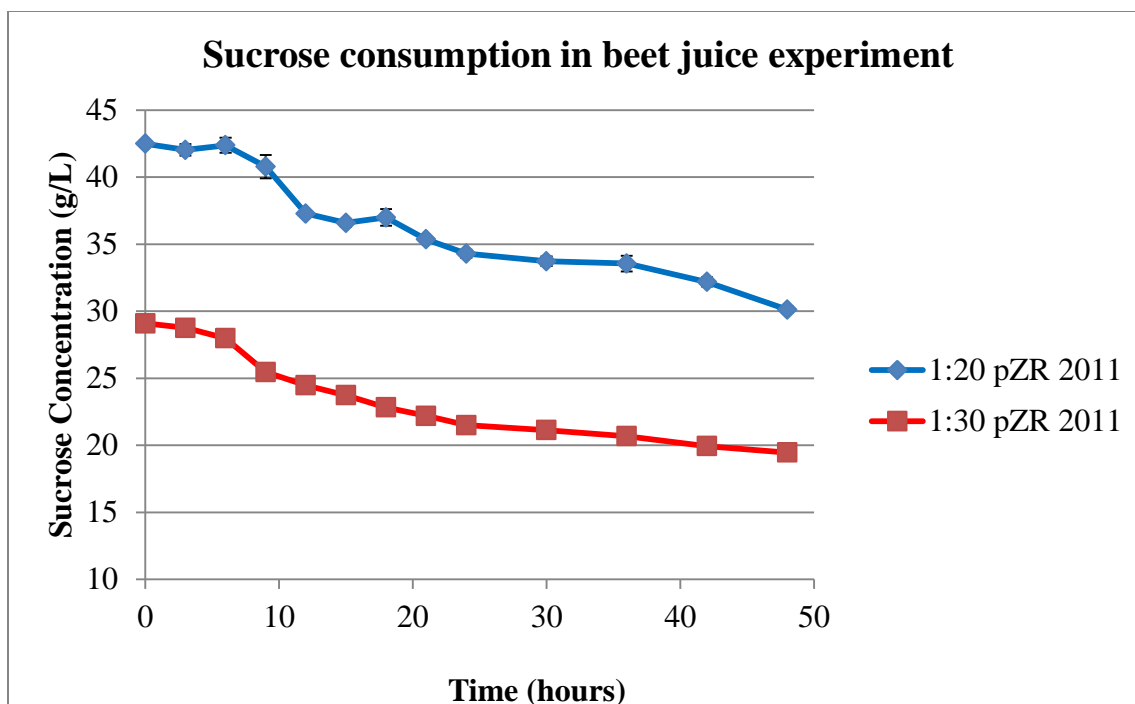


Figure 17: Sucrose Concentration. Sucrose concentration in *E. coli W* over 48 hours of growth.

3.4 Discussion

Introduction of pZR2011 (harboring genes *pdc* and *AdhB*) into *E. coli W* yielded ethanol production using beet juice concentrate as the sole carbon source. Total production of ethanol was highest in the 1:20 dilution but the highest productivity rate was observed in the 1:30 dilution. Sucrose consumption by S4335 indicated that as the ethanol increased the concentration of sucrose in the beet juice concentrate decreased. It has been already established that *E. coli W* contains sucrose transporters and genes that allow for the metabolism of sucrose (Akdağ & Çalık, 2015). Both concepts of the utilization of sucrose by *E. coli W* and the ethanol production observed in this study

suggests that the engineered strain S4335 of *E. coli* W can utilize the sucrose in the beet juice concentrate and convert it ethanol.

As mentioned previously, *Beta vulgaris* L. sugar beets could be used as a biofuel feedstock because of the very high sugar content and require low amount of processing unlike lignin-cellulose based biofuel feedstocks. The development of a long term storage solution in form of beet juice concentrate could alleviate issues with using this beet juice concentrate as a potential biofuel feedstock (Vargas-Ramirez et al., 2013). Improvement of bioethanol production from the S4335 of *E. coli* W using the beet juice concentrate as a sole carbon source could lead to a new avenue of bioethanol production from *Beta vulgaris* L. Since sugar beets require less processing and higher sugar content than corn starch and higher productivities by acre, sugar beets could be economically viable to use as a biofuel feedstock. Additional improvements would be required to increase ethanol production in S4335 of *E. coli* W which could be accomplished by increasing expression of genes *pdc* and *adhB* so that high quantities of ethanol could be produced rapidly. This development allows for a potential avenue to become available that would allow for the usage of long term storage of beet juice concentrate to convert to ethanol biofuel.

Chapter 4: Engineering of cyanobacteria to produce fuel ethanol using CO₂ and water

ABSTRACT

Current use of fossil fuels is both expensive and detrimental to the environment. Renewable fuels produced from plant biomass are not currently efficient enough to meet national demands and compete with other crops for farmable land that is required for food. Current ethanol plants release one-third of the carbon as CO₂ during fermentation. For example, a 100 MGY (million gallons per year) ethanol plant releases over 23 tons/hr of CO₂. Ideally, a photosynthetic organism could be engineered to convert the waste CO₂ back into fuel ethanol. The use of cyanobacteria provides such an opportunity. Cyanobacteria have minimal nutrient requirements and do not use arable land for growth. Additionally, cyanobacteria can be genetically engineered to directly convert CO₂ and H₂O into ethanol. Using cyanobacteria to produce ethanol bypasses the need to create extensive infrastructure to ferment and process plant biomass into usable biofuel.

In this study, two genes encoding pyruvate decarboxylase (*pdh*) and alcohol dehydrogenase II (*adhB*) were introduced to cyanobacteria. These two genes are required for converting endogenous pyruvate to ethanol. The transgenic cyanobacteria were confirmed to produce ethanol using only CO₂, mineralized H₂O and sunlight. Further work will attempt to increase the production of ethanol to a commercial viable level. If successful, the CO₂-to-ethanol model could allow for the replacement of fossil fuels with a renewable ethanol fuel.

4.1 Introduction

Growing greenhouse gas emissions from the burning of fossil fuels and the growing energy demand has put massive strain on the environment. As the public increasingly invests in more renewable energy sources more interest has been focused on biofuel development. Currently the majority of the energy production is reliant on fossil fuels but energy production from renewable resources such as biofuels could help alleviate climate change.

In the United States most biofuel production is reliant on first generation biofuel production which primary uses corn as the primary biofuel feedstock or soybeans for biodiesel (de Vries et al., 2010). One of the main arguments against first generation biofuels is the food vs fuel argument in which farmable land is being used to create biofuel feedstocks rather than food for consumption (Odling-Smee, 2007). Another disadvantage of first generation biofuels is the high processing costs and that the consumption of fossil fuels is still required to create and transport the final biofuel product (Fargione et al., 2008).

Fourth generation biofuels alleviate many of these concerns by focusing on the production of the biofuel or high-value compound directly from the organism. This biofuel production strategy would allow for microalgae species to directly create “drop in biofuel” which could be directly harvested from the media of the culture. “Drop in biofuels” would allow for complete compatibility with conventional gasoline engines (Xianhai Zenga, 2011).

The use of microalgae is an attractive host for fourth generation biofuels. Microalgae are much easier to genetically modify than plants and the most used microalgae have been fully sequenced (Johnson et al., 2016). Another advantage of using microalgae is the simple nutrient requirements which are just CO₂, mineralized water, and light. These attributes of easier genetic manipulation and simple nutrient requirements of microalgae could allow for genetically modified mutants to directly produce biofuels from CO₂ and H₂O which would essentially create “cell factories” (Xianhai Zeng, 2011).

In this study, cyanobacteria *Anabaena* sp. PCC 7120 was genetically modified using conjugation to insert the genes *pdh* and *adhB* contained in a self-replicating plasmid that would allow for the production of ethanol. Only the two genes *pdh* and *adhB* which allows for the expression of proteins pyruvate decarboxylase and alcohol dehydrogenase II from *Zymomonas mobilis* would allow for the production of ethanol (Deng & Coleman, 1999).

4.2 Materials and Methods

4.2.1 Construction of pZR2011

pZR2011 (Table 10) was an ethanol producing plasmid created from pZR 672 (Table 10) and used pZR1188 (Table 10) as the backbone vector. pZR672 was digested using *XbaI* & *BamHI* to excise the fragment containing the ethanol producing genes (*pdh*, *AdhB*). The backbone pZR1188 was digested with *AvrII* & *BamHI* and then the fragment from pZR672 was then ligated into the shuttle vector pZR1188.

Table 10: Plasmid and Strain list for ethanol production from *Anabaena* 7120

Plasmid or strain	Relevant characteristic(s)**	Source or reference
Plasmids		
pZR2011	Shuttle Vector, 2 ethanol production genes (<i>pdc</i> , <i>adhB</i>); Nm ^r /Km ^r	This study
pZR1188	Shuttle Vector; Nm ^r /Km ^r	Halfmann et al. 2014***
pZR672	Shuttle vector; Ethanol producing plasmid containing 2 ethanol production genes (<i>pdc</i> , <i>adhB</i>); Nm ^r /Km ^r	Zhou et al. 2009 unpublished construction
pRL443	Conjugal plasmid; Amp ^r , Tc ^r	(Jeff Elhai, 1997)
pRL623	Helper plasmid; Cm ^r /Em ^r	(Jeff Elhai, 1997)
Bacterial Strains		
<i>E. coli</i> NEB10β	Cloning, conjugal transfer of shuttle plasmids into PCC7120	New England Biolabs
WT 7120	<i>Anabaena</i> sp. PCC 7120 wild-type strain	
S4335	<i>Anabaena</i> sp. PCC 7120; Positive mutant harboring pZR2011 genes <i>pdc</i> and <i>AdhB</i> with <i>psbA1</i> promoter; Nm ^r /Km ^r	This study
** Ap ^r , ampicillin resistance; Sp ^r /Sm ^r , spectinomycin-streptomycin resistance; Tc ^r , tetracycline resistance; Nm ^r /Km ^r , neomycin-kanamycin resistance; Cm ^r /Em ^r chloramphenicol-erythromycin resistance		

4.2.2 Conjugal Transfer of pZR2011 from *E. coli* to *Anabaena*

The transformation efficiency of plasmids from *E. coli* to cyanobacteria can be increased drastically if the cargo plasmid is pre-methylated (Jeff Elhai, 1997). Helper plasmid pRL623 containing methylase genes which encodes for M.AvaI, M.Eco47II, and M.EcoT22I, which protects against restriction cut sites of AvaI, AvaII, and AvaIII, respectively (Jeff Elhai, 1997). The cargo plasmid pre-methylated by the previously stated methylases remains intact in *mcrBC*⁻ *E. coli* strains, such as NEB10β which was used in this study. *E. coli* NEB10β containing pRL623 and conjugal plasmid pRL443 was

mated with *E. coli* NEB10 β cargo plasmid pZR2011. The resulting strain which contained pZR2011, pRL443, and pRL623 was subjected to mating with *Anabaena* sp. PCC 7120 (hereafter referred as PCC7120) which resulted in positive mutant (S4335), following standard protocols (Elhai & Wolk, 1988). All conjugations were performed as standard protocol with the following alterations (Elhai & Wolk, 1988). *E. coli* NEB10 β harboring the cargo plasmid pZR2011 was isolated on LB plates containing appropriate antibiotic. Selected colonies were inoculated in 2 mL of LB with corresponding antibiotic grown overnight at 37°C at 250 RPM. Cultures were subcultured by inoculating 200 μ L into 2 mL of LB plus appropriate antibiotic and grown for 3 additional hours until O.D._{600nm} reaches between 0.4 and 0.5. Cells were then centrifuged at 12,000 g for 1 minute and then washed 3 times using 1 mL of LB without antibiotics. After 3 washes the cells were resuspended in 150 μ L of LB and subsequently mated with the NEB10 β strain harboring pRL 443 and pRL 623 for 30 minutes. Preparation of PCC7120 was as follows, 10 mL of PCC7120 WT was grown until exponential phase (O.D._{700nm} = 0.3-0.5) and then sonicated (Branson 1510 water bath sonicator) for 60-120 seconds to break filaments into 1-3 cell lengths, confirmed by microscopy. Sonicated PCC7120 WT was then centrifuged at 4000 x g for 10 minutes to form a cell pellet. Pellet was resuspended in 200 μ L of BG-11 media to wash the culture. The culture was washed an additional 2 times using the centrifuge and resuspended in BG-11 media. Conjugation plates were prepared as BG-11 + agar with 5% LB added. Droplet conjugation plate method was used since all plasmids were not integrating into the chromosome. Conjugation plates were prepared by placing 5 μ L drops of the NEB10 β mating mixture onto Immobilon-nitrocellulose membrane (HATF08550, Millipore) and allowed to dry briefly. A serial dilution of

PCC7120 WT was created starting with 1:10 and ending with 1:100000. The dilutions were then pipetted on top of the NEB10 β mixture spots in 5 μ L aliquots. Conjugation plate was then placed in standard growth conditions for cyanobacteria at 30°C and incubated under continuous white-light (ca. 50 μ E M⁻² S⁻¹) for 24 to 48 hours. After initial incubation the nitrocellulose membrane was then placed onto BG-11 + Agar with appropriate antibiotic and incubated under the same growth conditions until antibiotic resistant colonies were observed (10-20 days). Isolated antibiotic resistant colonies were then picked and placed on BG-11 + agar with appropriate antibiotic. Subsequent colony PCR was performed to verify presence of new mutant strains using standard PCR program with one modification of preheating cyanobacterial colonies by heating the cell suspension in 10 μ L of ddH₂O at 95 °C for 10 minutes. Primer pair ZR1137 & ZR1139 were used for the colony PCR to verify mutant colonies after conjugation of pZR2011 for the ethanol producing genes (*pdh* & *adhB*) (Table 11).

Table 11: Primer list for ethanol production from *Anabaena* 7120.

Primer name	Oligonucleotide sequence (5' \rightarrow 3')*	Purpose
ZR45	CCTCGTAGAACTAGCAAAG	Used for verification of pZR2011, genes (<i>pdh</i> , <i>adhB</i>)
ZR453	TCTCGAGCTTGTCATCGTCATCCTTGTAATC	

4.2.3 Bacterial Strains and Growth Conditions

Bacterial strains used in this study listed in Table 10. All *E.coli* NEB10 β strains were grown at 37°C at 250 RPM in Lysogeny broth (LB). Cyanobacterial strains were grown in BG-11 media with nitrate. Cyanobacteria strains were incubated at 30°C and

agitated at 150 RPM in Innova-44R shaker (New Brunswick) under continuous white-light (ca. $50 \mu\text{E M}^{-2}\text{S}^{-1}$) illumination. One hundred $\mu\text{g/mL}$ of Nm was added to BG-11 for growth of appropriate mutant cyanobacteria strains.

4.2.4 Growth Curve and Ethanol Production Experiment

To analyze growth rate and ethanol production of S4335 a growth experiment was conducted. All previously stated cultures were adjusted to an O.D._{700 nm} of 0.4. After the O.D. was adjusted 100 μL of culture was inoculated into 30 mL of BG-11 and with appropriate antibiotic as seed cultures. After seed cultures reached an O.D._{700nm} between 0.5 and 0.7 seed cultures were adjusted again to an O.D._{700nm} of 0.4. After all cultures were adjusted experiment cultures were inoculated using 100 μL of adjusted seed culture to inoculate into 50 mL of BG-11 with appropriate antibiotics. Strains used in the experiment included S4335 in BG-11+ 100 $\mu\text{g/mL}$ Nm. Schedule of samples taken were conducted as listed in Table 15. O.D. readings were performed using 300 μL of culture aliquot into a Costar 96 (Corning™ mfr. No. 3615) well plate and O.D. readings were taken at 700 nm using the Plate Reader (BioTek Synergy 2 Multi-detection Microplate Reader). Medium samples were taken as 1mL aliquots and placed into 1.5mL eppendorf tubes. Medium samples were processed by centrifugation at 12,000 x g for 10 min. Supernatant was then removed and placed into a fresh eppendorf tube and frozen overnight at -20°C. Samples were then thawed at room temperature and then centrifuged again at 12,000 x g for 10 minutes. Supernatant from the medium samples were then passed through a 0.2 μm sterile filter and placed in a 2 mL HPLC vial for HPLC analysis.

4.2.5 HPLC Analysis of ethanol production

An Agilent HPLC (1220 Infinity II LC System) with a refractive index detector was used. Reaction products and standard compounds were detected by absorbance at 260 nm. A RHM-monosacchride H⁺ column was used (Rezex 7.8mm × 250mm, Rezex) was equilibrated at a flow rate of 0.6 ml min⁻¹ with ddH₂O water buffer. An isocratic program with a runtime of 25 min was used. Ethanol concentration was identified through the use of an ethanol standard curve and a retention time of 22 minutes.

4.2.6 Chlorophyll Extraction and Analysis of Photosynthetic Activity

Chlorophyll was extracted using a 90% methanol extraction procedure described previously (Meeks & Castenholz, 1971). Oxygen evolution was quantified in 1 mL culture samples that were normalized for O.D._{700nm} of 1.0 in BG-11 with 1mM NaHCO₃. Oxygen evolved was detected and analyzed using a Clark-type electrode and DW2 Oxygen Electrode Chamber with O₂ View Oxygen Monitoring software (Hansatech). Light was provided to the samples using an LS2/H Tungsten-halogen 50W light source and adjusted with neutral density filters. Light intensity and sample temperature was monitored using a Quantitherm light-temperature meter during experimentation. All measurements included three replicates to ensure accuracy.

4.3 Results

4.3.1 Verification of positive mutants bearing pZR2011

Confirmation of *Anabaena* 7120 mutants was completed using colony Polymerase chain reaction (cPCR). Primers used to verify pZR2011 included ZR45 and ZR453 (Figure 18). Positive conjugates were further purified by restreaking it on the same

antibiotic plate for segregation and the final mutant strain containing pZR2011 was designated S4335.

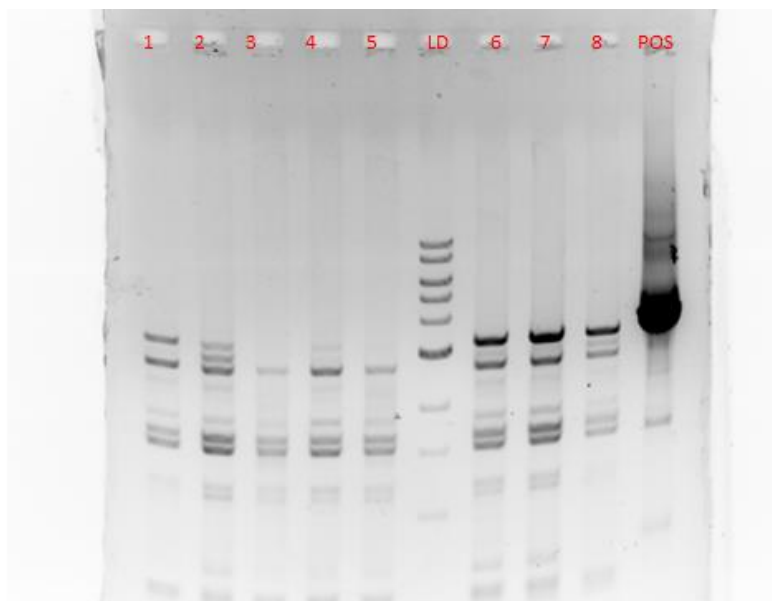


Figure 18: Colony PCR of colonies of *Anabaena* 7120. Colonies were positive for the ~3.3 kb band (genes *adhB* and *pdc*). Colonies 1, 6, 7 were all positive for the insert. Positive control and ladder are listed as (Pos) and (LD), respectively.

4.3.2 Growth Experiment

Growth experiment was performed on S4335 bearing pZR2011 and wild-type (Figure 19). Growth experiment showed best growth was achieved by wild-type followed by S4335 bearing pZR2011.

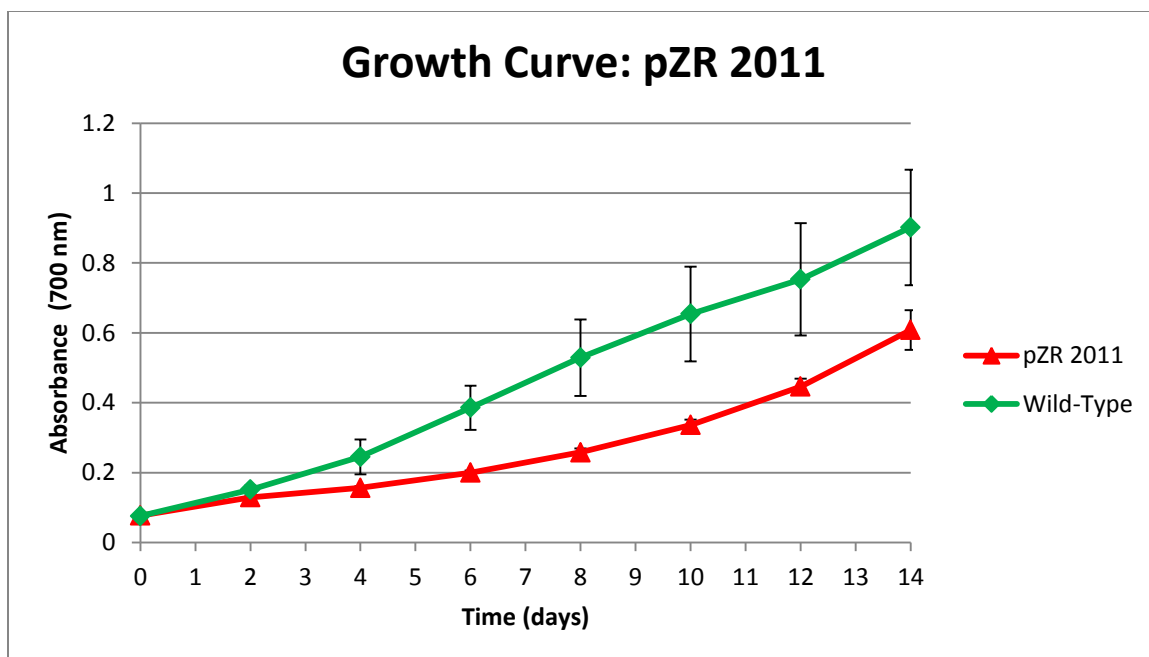


Figure 19: Growth Curve of S4335 vs. wild-type. Growth experiment included mutant strain S4335 bearing pZR2011 and wild-type as control.

4.3.3 Photosynthetic Activity

Photosynthetic efficiency was calculated for all cultures included in the growth experiment (Figure 20). The cultures in the experiment included mutant strain S4335 bearing pZR2011 and wild-type. The photosynthetic efficiency of S4335 was 1.60-fold higher (at $200 \mu\text{mol m}^{-2} \text{s}^{-1}$), 1.16-fold higher (at $800 \mu\text{mol m}^{-2} \text{s}^{-1}$) than that of wild-type.

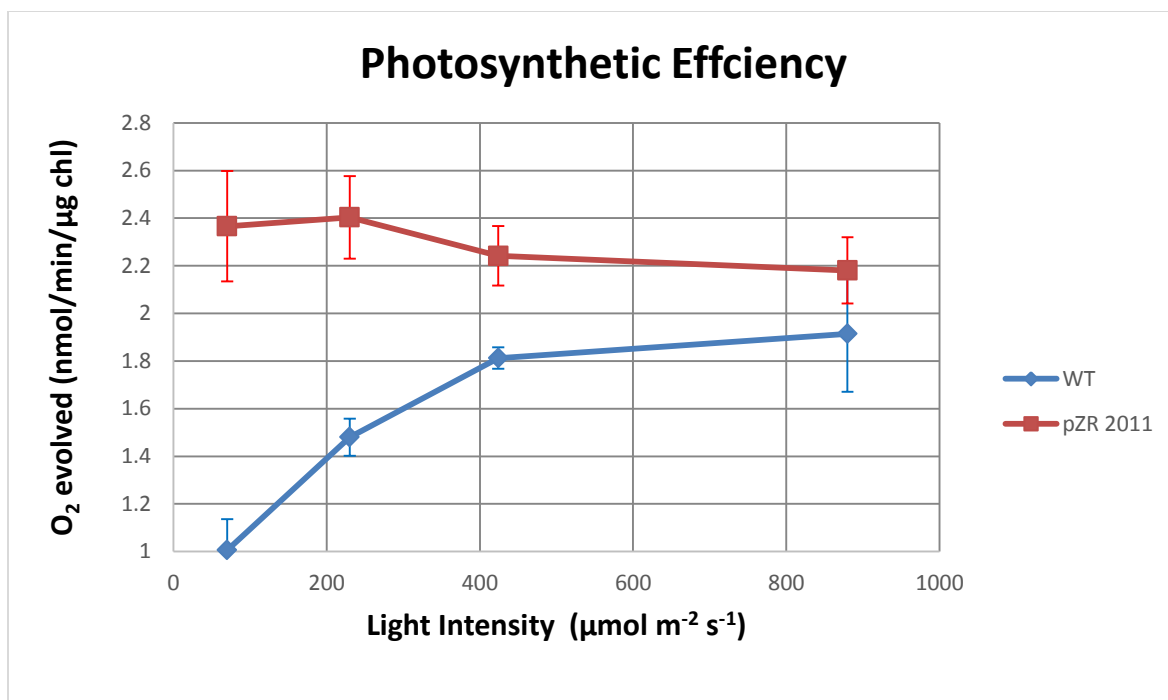


Figure 20: Photosynthetic activity of WT, S4335 mutant bearing pZR2011. All cultures were performed on species *Anabaena* PCC 7120. 1-mL aliquots (normalized at O.D._{700nm} of 1.0) in BG11 + 1mM NaHCO₃ were measured for oxygen evolution in four different light intensities 70, 230, 424, and 880 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

4.3.4 Ethanol Production

Ethanol production was measured according to the directions dictated in materials and methods section. There was only a single ethanol spike observed on one day in S4335 but no consistent production (Figure 21). Standard curve was processed before each batch of samples to ensure that instrument error was not the cause for the absence of ethanol.

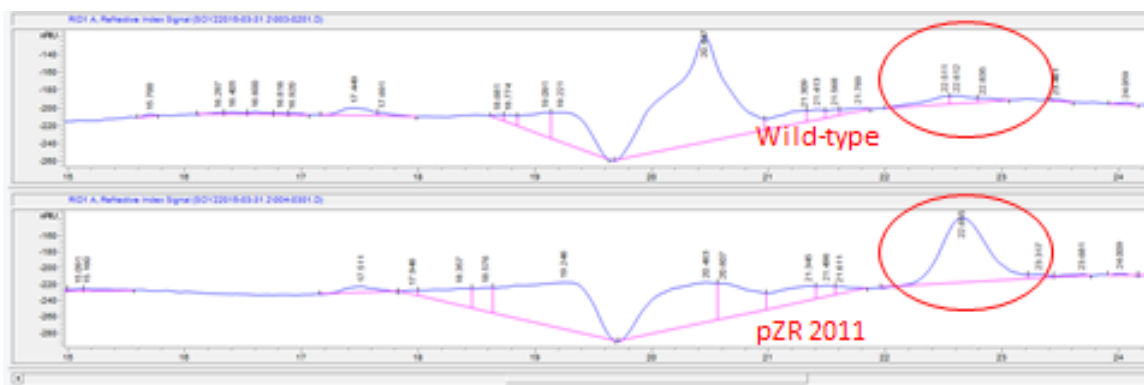


Figure 21: Ethanol Peak of S4335. Chromatograph of Refractive Index Detector (RID) on the HPLC of the ethanol peak detected in S4335 bearing pZR2011 vs wild-type. Ethanol peak elution at ≈ 20 min.

4.4 Discussion

Engineering cyanobacteria to produce fuel ethanol from CO_2 bypasses extensive infrastructure for fermenting and processing biomass-based biofuels. In addition, the use of fixed nitrogen sources (e.g., ammonium fertilizers for biomass/crop growth) increases production costs and harms the environment. *Anabaena* 7120 is naturally able to fix atmospheric N_2 , converting it to biologically assessable ammonium (NH_3). Thus, *Anabaena* 7120 is an attractive production vessel for biofuels with its simple nutrient requirements and promising output potential.

Two genes required for ethanol production were inserted into cyanobacteria replication vector pZR1188. The vector pZR1188 included two promoters *P_{nir}*, which is a nitrate inducible promoter, and *P_{psbA1}*, which is a constitutively active in *Anabaena* 7120. The two genes *pdh* and *adhB* were excised from pZR672 and then ligated to pZR1188 to form pZR2011. This allowed for the production of ethanol in the absence of nitrate since the *Anabaena* 7120 can fix nitrogen.

The results show that pZR2011 was successfully confirmed via cPCR indicating that the plasmid was successfully conjugated into *Anabaena* 7120. The results of the growth experiment show that positive mutant S4335 bearing pZR2011 showed slower overall growth compared to wild-type. However, photosynthetic activity suggested that the mutant had a higher photosynthetic potential compared to wild-type. Although consistent ethanol production was not found in the mutant containing pZR2011, ethanol peaks were still detected that suggest that this mutant is capable of producing ethanol. The inconsistent production of ethanol is thought to be restricted in the metabolic pathway most likely due to the availability of the precursor molecule pyruvate. The overall the results of this study suggest ethanol production from *Anabaena* 7120 is possible but some potential metabolic bottlenecks are present that are interfering with consistent ethanol production from pZR2011.

Future experiments will attempt to increase the production rate of ethanol from *Anabaena* 7120 by the addition of a synthetic photo-respiratory bypass pathway. This photo-respiratory bypass will allow for an extra pathway for *Anabaena* 7120 to potentially increase CO₂ fixation and the production of ethanol.

Chapter 5: Introduction of a Synthetic CO₂ Fixing Photorespiratory Bypass into *Anabaena* PCC 7120

ABSTRACT

The biosphere's photosynthetic productivity is limited by the enzymatic activity of Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO). The majority of the global assimilation of CO₂ into organic carbon compounds is performed by RubisCO. RubisCO has difficulty discriminating between CO₂ and O₂ which can lead to photorespiration and results in the loss of fixed carbon and nitrogen. In this study the 3-hydroxypropionate synthetic photorespiratory bypass (3-HPA pathway) was introduced into *Anabaena* PCC 7120. The 3-HPA photorespiratory bypass was introduced in an attempt to provide an additional CO₂-fixing pathway that would supplement the Calvin-Benson cycle. It was found that the introduction of the 3-HPA bypass marginally increased photosynthetic activity but inhibited growth rates of the mutant strains. This study has shown implications that increased photosynthesis through the use of synthetic biology is attainable. However, more research and genetic manipulation will be required to allow for production of "green" high value compounds using supplementary synthetic CO₂-fixing pathways.

5.1 Introduction

Oxygenic photosynthesis is the most important biochemical process to convert solar energy to chemical energy. Oxygenic photosynthesis requires RubisCO to fix CO₂ to sugars. However, RubisCO has both carboxylase and oxygenase activities and the specificity between O₂ and CO₂ is fairly low, which can lead to photorespiration and ultimately the loss of 25% fixed carbon (Yonatan Savir, 2010). One of the highest priorities in photosynthesis research is to engineer a powerful RubisCO to increase CO₂ fixation and reduce photorespiration activity. In previous studies RubisCO has been reengineered but only limited success was achieved. However, new avenues of engineered RubisCO are being investigated (Christoph Peterhansel, 2008). It has been shown that serious attempts to engineer RubisCO are limited with constraints in both biochemical and abiotic factors. In addition, it has been theorized that the efficiency of RubisCO can only be improved marginally as most organism's RubisCO has been adapted to its specific intracellular environment and the net photosynthesis rate is already nearly optimal (Yonatan Savir, 2010). Due to the great complexity of engineering RubisCO and the numerous constraints already present, it may be more prudent to instead improve net photosynthesis rate through the use of photorespiratory bypasses (Shih et al., 2014).

Adding to the complexities between RubisCO and its oxygenase and carboxylase activities, it has been found that during photorespiration toxic intermediate 2-phosphoglycolate is formed. Although 2-phosphoglycolate is a toxic intermediate, it is recycled by the C₂ photorespiratory cycle. However, the process of recycling this

intermediate has a high cost of ATP and requires the use of over a dozen enzymes and transporters (Kleczkowski, 1992).

The introduction of a synthetic carbon fixation pathway in an organism could allow for potential increase in both photosynthetic efficiency and a way to avoid the complexities involved with increasing efficiency of the C_2 cycle (Blankenship et al., 2011). Out of the six known carbon fixation cycles only the 3-hydroxypropionate (3-HPA) pathway so far has been found to be completely oxygen insensitive (G. Fuchs, 2011). This carbon fixation pathway was first characterized in *Chloroflexus aurantiacus* which uses this cycle for autotrophic CO_2 fixation (G. Fuchs, 2011). In addition, using this pathway as a synthetic carbon fixation pathway has added benefits because all the enzymes have been characterized (G. Fuchs, 2011). The 3-HPA pathway begins with the use of ATP and biotin-dependent acetyl-CoA and propionyl-CoA carboxylases, which act as carboxylating enzymes and produce bicarbonate and glyoxylate. The second portion of this cycle utilizes glyoxylate and fixes an additional bicarbonate and produces pyruvate (G. Fuchs, 2011).

Previously a study introduced the 3-HPA into *Synechococcus elongatus* sp. PCC 7942 but found no increase in growth rate (Shih et al., 2014). This study will investigate the introduction of the 3-HPA pathway into an ethanol producing strain of cyanobacterium *Anabaena* sp. strain PCC 7120 (hereinafter referred to as *Anabaena* 7120). *Anabaena* 7120 is a photosynthetic prokaryote that is capable of photosynthesis and solar-powered nitrogen fixation through specialized heterocysts (Flores & Herrero, 2010). *Anabaena* 7120 has been shown previously that it can be engineered to produce high-value compounds including farnesene and limonene (C. Halfmann et al., 2014).

Anabaena 7120 could be more economically advantageous than unicellular strains because it does not require fixed nitrogen which could help alleviate costs associated with growth media. This benefit would be most noticed in the production of biofuel when scaling up to industrial levels.

It is hypothesized that the addition of an additional carbon fixation cycle (3-HPA) could increase CO₂ fixation rate and allow for more pyruvate production. Therefore, more pyruvate can be converted to ethanol. The addition of the 3-HPA pathway could also act as a photorespiratory bypass allowing an increase photosynthetic efficiency.

Previous studies on ethanol production in cyanobacteria have relied on *Synechococcus elongatus* sp. PCC 7942 and *Synechococcus* sp. PCC 6803 which are unicellular cyanobacteria that do not fix nitrogen (Dexter, Armshaw, Sheahan, & Pembroke, 2015). Although significant strides have been accomplished in increasing ethanol production the same problems of low yields and productivities are bottlenecks. The productivity is consistently lower when comparing these productivities to the native heterotrophic processes (Dexter et al., 2015).

In this study the genes required for the second half of the 3-HPA bicycle (using glyoxylate to produce bicarbonate and pyruvate) were subcloned into a replicating plasmid in an attempt to increase CO₂ fixation rate and intracellular pyruvate concentration. Introduction of a metabolic pathway that increases an endogenous metabolite could be used as an avenue to produce biofuel (P. Savakis et al., 2015). Introducing the 3-HPA metabolic pathway into *Anabaena* 7120 could allow for increased efficiency in the production of ethanol through the use of the genes pyruvate

decarboxylase (*pdc*) and alcohol dehydrogenase II (*adhB*). Pdc uses pyruvate and produces Acetyl-CoA which is then used by AdhB to create ethanol. A mutant strain of *Anabaena* 7120 containing both the 3-HPA pathway and genes *pdc* and *adhB* could allow for the efficient production of ethanol from cyanobacteria that only requires CO₂, H₂O, and sunlight.

5.2 Materials and Methods

5.2.1 Construction of pZR2093 & pZR2053

To follow more simply a table of the construction pathway used for the creation of pZR2093 and pZR2053 refer to (Table 12). Vector pZR2050 (Table 13) was created using pZR670 (Table 13) as a backbone vector. A polylinker containing *Bam*HI-*Bst*BI-*Eco*NI-*Stu*I-*Avr*II-*Zra*I-*Aat*II-*Bsi*WI-*Xho*I multiple cloning sites (MCS) was created using annealed oligos ZR1293 and ZR1294 (Table 14). DNA fragment created contained 5' overhangs and was ligated into *Bam*HI & *Nsi*I digested pZR670 to create pZR2050. Construction of pZR2054 (Table 13) was created using pZR2050 as a backbone vector and inserted the 4 gene fragments from pNS1-012 . pNS1-012 (Table 13) was a plasmid provided by Matthew Mattozzi from Harvard Wiess institute which contained genes β -methylmalyI-CoA dehydratase (*Mch*) (Caur_0173), mesaconyl-C4-CoA hydratase (*Meh*) (Caur_0180), mesaconyl-C1-CoA-C4-CoA transferase (*Mct*) (Caur_0175), and L-malyI CoA lyase (*MclA*) (Caur_174). pNS1-012 was digested using *Not*I at 37°C. Digestion was then heat inactivated at 65°C for 20 minutes. After heat inactivation the plasmid containing the 5' overhangs produced by *Not*I were filled in using T4 DNA Polymerase at 37°C for 30 minutes creating a blunt end. Digestion reaction containing T4 DNA

polymerase was heat inactivated again at 65°C for 20 minutes. After final heat inactivation the digestion reaction was digested with the second enzyme *Bgl*III at 37°C to isolate the 4 gene fragments from pNS1-012. The backbone vector pZR2050 was digested using *Bam*HI & *Stu*I. Gene fragments from pNS1-012 containing genes *Mch*, *Meh*, *Mct*, *MclA* was then ligated it into *Bam*HI & *Stu*I digested pZR2050 to create pZR2054. pZR2011 (Table 13) was an ethanol producing plasmid created from pZR672 (Table 13) and used pZR1188 (Table 13) as the backbone. pZR672 was digested using *Xba*I & *Bam*HI to excise the fragment containing the ethanol producing genes (*pdh*, *AdhB*). The fragment from pZR672 was then ligated into *Avr*II & *Bam*HI digested pZR1188 to produce pZR2011.

pZR2091 (Table 13) was a construction plasmid used for the final construction of pZR2093. pZR2091 was created by the removal of a fragment from the MCS of pZR2011 by digestion of *Zra*I & *Sma*I and then religated back together to produce pZR2091. pZR2092 was a construction plasmid which used TOPO Cloning kit as a backbone and inserted PCR fragment from pZR2091. To create pZR2092 (Table 13), pZR2091 was used for PCR amplifying ethanol production genes pyruvate decarboxylase (*pdh*) and alcohol dehydrogenase II (*adhB*) using ZR1137 & ZR1138 (Table 14) to create a 4458bp fragment which was ligated into pCR®2.1-TOPO® vector (TOPO TA Cloning® kit, Invitrogen) to produce pZR2092. pZR2093 (Table 13) which contained 4 genes from the 3-HPA bicycle (*mch*, *meh*, *mct*, *mclA*) and 2 ethanol production genes (*pdh*, *adhB*) was created using pZR2054 as a backbone vector and gene fragment containing ethanol production genes from pZR2092. Fragment from pZR2092 was created by

digestion using *AatII* & *SalI*. The 4.4kb fragment was then ligated into *AatII* & *XhoI* digested pZR2054 to create pZR2093.

pZR2053 was an expression vector containing 2 genes for the 3-HPA cycle malonyl-CoA reductase (Mcr) and propionyl-CoA synthase (Pcs). Construction of pZR2053 used pNIR as the backbone vector and p62-MP which was a plasmid provided by Matthew Mattozzi which contained genes malonyl-CoA reductase (Mcr) and propionyl-CoA synthase (Pcs). p62-MP was digested using *EcoRI* & *XhoI* creating the fragment containing genes *mcr* & *pcs*. Shuttle vector pNIR was digested with *EcoRI* & *XhoI* and then the fragment from p62-MP was ligated into *EcoRI* & *XhoI* digested pNIR to produce pZR2053.

Table 12: Construction pathway for pZR2093 and pZR2053

Plasmid or strain	Relevant characteristic(s)*	Source or reference
Plasmids		
pZR2050	created using pZR670 as a backbone vector with polylinker consisting of annealed oligos ZR1293 and ZR1294 containing <i>BamHI</i> - <i>BstBI</i> - <i>EcoNI</i> - <i>StuI</i> - <i>AvrII</i> - <i>ZraI</i> - <i>AatII</i> - <i>BsiWI</i> - <i>XhoI</i> multiple cloning sites (MCS)	This Study
pZR2054	created using pZR2050 as a backbone vector and inserted the 4 gene fragments from pNS1-012. Genes <i>mch</i> , <i>meh</i> , <i>mct</i> , <i>mclA</i> were isolated using digestion of <i>NotI</i> and <i>BglII</i> . 4 genes were religated into <i>BamHI</i> & <i>StuI</i> cut pZR2050	This Study
pZR2011	created from pZR672 and used pZR1188 as the backbone vector. pZR672 was digested using <i>XbaI</i> & <i>BamHI</i> to excise the fragment containing the ethanol producing genes (<i>pdh</i> , <i>AdhB</i>) on pZR 672 and religated into pZR1188	This Study
pZR2091	was created from pZR2011 by removing the fragment from the MCS using <i>ZraI</i> & <i>SmaI</i>	This Study
pZR2092	Created by using TOPO Cloning kit as a backbone vector and inserted PCR fragment from pZR2091 using ZR1137 & ZR1138	This Study
pZR2093	was created using pZR2054 as a backbone vector cut with <i>AatII</i> & <i>XhoI</i> which contained genes <i>mch</i> , <i>meh</i> , <i>mct</i> , <i>mclA</i>	This Study

	. Gene fragment was removed from pZR2092 using <i>AatII</i> & <i>SalI</i> containing genes <i>pdh</i> and <i>adhB</i> . Genes were then religated into pZR2054	
pZR2053	created using backbone vector pNIR cut by <i>EcoRI</i> & <i>XhoI</i> . Genes <i>pcs</i> and <i>mcr</i> were digested from p62-MP using <i>EcoRI</i> & <i>XhoI</i> and then religated into pNIR	This Study
pNS1-012	Plasmid containing genes <i>mch</i> , <i>meh</i> , <i>mct</i> , <i>mclA</i>	(Mattozzi, Ziesack, Voges, Silver, & Way, 2013)
p62-MP	Plasmid containing genes <i>pcs</i> and <i>mcr</i>	(Mattozzi et al., 2013)
pNIR	Expression vector for <i>Anabaena</i> 7120	Zhou et al.

5.2.2 Conjugal transfer of plasmids from *E. coli* to *Anabaena*

The transformation efficiency of plasmids from *E. coli* to cyanobacteria can be increased drastically if the cargo plasmid is pre-methylated (Jeff Elhai, 1997). Helper plasmid pRL623 containing methylase genes which encodes for M.AvaI, M.Eco47II, and M.EcoT22I, which protects against restriction cut sites of AvaI, AvaII, and AvaIII, respectively (Jeff Elhai, 1997). The cargo plasmid pre-methylated by the previously stated methylases remains intact in *mcrBC⁻* *E. coli* strains, such as NEB10 β which was used in this study. *E. coli* NEB10 β containing pRL623 and conjugal plasmid pRL443 was mated with *E. coli* NEB10 β cargo plasmid pZR2011. The resulting strain which contained pZR2011, pRL443, and pRL623 was subjected to mating with *Anabaena* sp. PCC 7120 (hereafter referred as PCC7120) which resulted in positive mutant (S4335), following standard protocols (Elhai & Wolk, 1988). *E. coli* NEB10 β strains containing pZR2093 along with pRL 443 and pRL623 plasmids were mated with PCC7120 which resulted in S4412. Construction of PCC7120 containing both pZR2093 and pZR2053 was performed as follows. *E. coli* NEB10 β containing pZR2053 was mated with a NEB10 β

strain harboring pRL443 and pRL623. The resulting strain harboring pZR2053, pRL443, and pRL623 was then mated with positive mutant strain S4412 (PCC7120) conjugation performed according to standard protocols (Elhai & Wolk, 1988). Two positive mutants were confirmed harboring both pZR2093 and pZR2053 denoted as S4630 and S4631. All conjugations were performed as standard protocol with the following alterations (Elhai & Wolk, 1988). *E. coli* NEB10 β harboring the cargo plasmid (pZR2093 or pZR2011) was isolated on LB plates containing appropriate antibiotic. Selected colonies were inoculated in 2 mL of LB with corresponding antibiotic grown overnight at 37°C at 250 RPM. Cultures were subcultured by inoculating 200 μ L into 2 mL of LB plus appropriate antibiotic and grown for 3 additional hours until O.D._{600nm} reached between 0.4 and 0.5. Cells were then centrifuged at 12,000 g for 1 minute and then washed 3 times using 1 mL of LB without antibiotics. After 3 washes the cells were resuspended in 150 μ L of LB and subsequently mated with the NEB10 β strain harboring pRL 443 and pRL 623 for 30 minutes. Preparation of PCC7120 was as follows, 10 mL of PCC7120 WT was grown until exponential phase (O.D._{700nm} = 0.3-0.5) and then sonicated (Branson 1510 water bath sonicator) for 60-120 seconds to break filaments into 1-3 cell lengths, confirmed by microscopy. Sonicated PCC7120 WT was then centrifuged at 4000 x g for 10 minutes to form a cell pellet. Pellet was resuspended in 200 μ L of BG-11 media to wash the culture. The culture was washed an additional 2 times using the centrifuge and resuspended in BG-11 media. Conjugation plates were prepared as BG-11 + agar with 5% LB added. Droplet conjugation plate method was used since all plasmids were not integrating into the chromosome. Conjugation plates were prepared by placing 5 μ L drops of the NEB10 β mating mixture onto Immobilon-nitrocellulose membrane (HATF08550,

Millipore) and allowed to dry briefly. A serial dilution of PCC7120 WT was created starting with 1:10 and ending with 1:100000. The dilutions were then pipetted on top of the NEB10 β mixture spots in 5 μ L aliquots. Conjugation plate was then placed in standard growth conditions for cyanobacteria at 30°C and incubated under continuous white-light (ca. 50 μ E M⁻² S⁻¹) for 24 to 48 hours. After initial incubation the nitrocellulose membrane was then placed onto BG-11 + Agar with appropriate antibiotic and incubated under the same growth conditions until antibiotic resistant colonies were observed (10-20 days). Isolated antibiotic resistant colonies were then picked and placed on BG-11 + agar with appropriate antibiotic. Subsequent colony PCR was performed to verify presence of new mutant strains using standard PCR program with one modification of preheating cyanobacterial colonies by heating the cell suspension in 10 μ L of ddH₂O at 95 °C for 10 minutes. Primer pair ZR1137 & ZR1139 were used for the colony PCR to verify mutant colonies after conjugation of pZR2093 for the ethanol producing genes (*pdh* & *adhB*). Primer pair ZR1305 & ZR1468 were used for the verification of the 4 genes in the 3-HPA pathway (*mch*, *meh*, *mct*, *mclA*) contained in pZR2093. Primer pair ZR45 & ZR22 were used for the colony PCR verification of pZR2053.

5.2.3 Bacterial strains and Growth Conditions

Bacterial strains used in this study listed in Table 13. All *E. coli* NEB10 β strains were grown at 37°C at 250 RPM in Lysogeny broth (LB). Cyanobacterial strains were grown in BG-11 media. Cyanobacteria strains were incubated at 30°C and agitated at 150 RPM in Innova-44R shaker (New Brunswick) under continuous white-light (ca. 50 μ E M⁻² S⁻¹) illumination. One hundred μ g/mL of Nm was added to BG-11 for growth of

appropriate mutant cyanobacteria strains. Ten µg/mL of Em was used for growth of mutant strains in BG-11.

Table 13: Plasmid and Strain list for 3-HPA bypass

Plasmid or strain	Relevant characteristic(s)**	Source or reference
Plasmids		
pZR670	Shuttle Vector Cm ^r /Em ^r	(Xu, Gu, He, & Zhou, 2015)
pZR2050	Oligos ZR1293 and ZR1294 were annealed, forming a MCS, and then cloned into <i>Bam</i> HI & <i>Nsi</i> II digested pZR670; Cm ^r /Em ^r	This study
pNS1-012	Plasmid containing 4 genes in the 3-HPA bicycle: β-methylmalyl-CoA dehydratase (<i>Mch</i>) (Caur_0173), mesaconyl-C4-CoA hydratase (<i>Meh</i>) (Caur_0180), mesaconyl-C1-CoA-C4-CoA transferase (<i>Mct</i>) (Caur_0175); Sp ^r /Sm ^r	(Mattozzi et al., 2013)
pZR2054	Plasmid used for construction of pZR2093	This study
pZR2091	Construction plasmid containing ethanol production genes pyruvate decarboxylase (<i>pdh</i>) and alcohol dehydrogenase II (<i>Adh</i> B); Nm ^r /Km ^r ,	This study
pZR2092	TOPO Vector containing ethanol production genes pyruvate decarboxylase (<i>pdh</i>) and alcohol dehydrogenase II (<i>Adh</i> B); Nm ^r /Km ^r & Ap ^r	This study
pZR2093	Shuttle Vector, 4 genes from the 3-HPA bicycle (<i>mch</i> , <i>meh</i> , <i>mct</i> , <i>mclA</i>) and 2 ethanol production genes (<i>pdh</i> , <i>adh</i> B); Cm ^r /Em ^r	This study
pZR1188	Shuttle Vector; Nm ^r /Km ^r	(C. Halfmann, L. Gu, & R. Zhou, 2014)
pZR672	Shuttle vector; Ethanol producing plasmid containing 2 ethanol production genes (<i>pdh</i> , <i>adh</i> B); Nm ^r /Km ^r	(Zhou R., 2009)
pZR2053	Shuttle vector; 2 genes for the 3-HPA bicycle malonyl-CoA reductase (<i>Mcr</i>) and propionyl-CoA synthase (<i>Pcs</i>); Nm ^r /Km ^r	This study
p62-MP	Expression vector containing 2 genes for the 3-HPA bicycle malonyl-CoA reductase (<i>Mcr</i>) and propionyl-CoA synthase (<i>Pcs</i>); Tc ^r	(Mattozzi et al., 2013)
pNIR	Shuttle Vector; Cm ^r /Em ^r	Zhou et al.

pRL443	Conjugal plasmid; Amp ^r , Tc ^r	(Jeff Elhai, 1997)
pRL623	Helper plasmid; Cm ^r /Em ^r	(Jeff Elhai, 1997)
Bacterial Strains		
<i>E. coli</i> NEB10β	Cloning, conjugal transfer of shuttle plasmids into PCC7120	New England Biolabs
WT 7120	<i>Anabaena sp.</i> PCC 7120 wild-type strain	
<i>E. coli</i> TOP10	Cloning host	Invitrogen
S4335	<i>Anabaena sp.</i> PCC 7120; Positive mutant harboring pZR2011 genes <i>pdc</i> and <i>AdhB</i> with <i>psbA1</i> promoter; Nm ^r /Km ^r	This study
S4412	<i>Anabaena sp.</i> PCC 7120; Positive mutant containing pZR2093 genes <i>pdc</i> and <i>AdhB</i> and 3-HPA bicycle genes (<i>mch,meh,mct,mclA</i>); Cm ^r /Em ^r	This study
S4630	<i>Anabaena sp.</i> PCC 7120; Positive mutant containing pZR2093 genes <i>pdc</i> and <i>AdhB</i> and 3-HPA bicycle genes (<i>mch,meh,mct,mclA</i>) also contains pZR2053 harboring genes (<i>pdc, mcr</i>) from 3-HPA Bicycle; Nm ^r /Km ^r ,	This Study
S4868	<i>Anabaena sp.</i> PCC 7120; Positive mutant containing pZR2093 genes <i>pdc</i> and <i>AdhB</i> and 3-HPA bicycle genes (<i>mch,meh,mct,mclA</i>) also contains pZR2053 harboring genes (<i>pdc, mcr</i>) from 3-HPA Bicycle; Nm ^r /Km ^r ,	This Study
S557	<i>Anabaena sp.</i> PCC 7120; Positive mutant harboring pZR672 genes <i>pdc</i> and <i>AdhB</i> with pNIR promoter; Nm ^r /Km ^r	This Study
** Ap ^r , ampicillin resistance; Sp ^r /Sm ^r , spectinomycin-streptomycin resistance; Tc ^r , tetracycline resistance; Nm ^r /Km ^r , neomycin-kanamycin resistance; Cm ^r /Em ^r chloramphenicol-erythromycin resistance		

Table 14: Primer list for 3-HPA bypass

Primer name	Oligonucleotide sequence (5' → 3')*	Purpose
ZR1293	TATGggatccttcgaaAGGCCTAGGGAcgtcgtacgctcga	Introduction of MCS into pZR2050
ZR1294	GATCtcgagcgtacgacgTCCCTAGGCCTttcgaaggatccCATATGCA	
ZR1137	tcctaggcggcCGCTCAGTGGAACGAAACTC	Used for PCR

		amplification of pZR2091 for the construction of pZR2092
ZR1138	TcctaggaTCCTGTTTTGAAATTAGAAAGCG	
ZR1137	tcctaggcggcCGCTCAGTGGAACGAAAACCTC	Used for verification of pZR2093, genes (<i>pdh</i> , <i>AdhB</i>)
ZR1139	CCGGCTAAAATCGATCACGTG	
ZR1305	CAATGACAACCCCAACAAGAAGTC	Used for verification of pZR2093, genes (<i>mch</i> , <i>meh</i> , <i>mct</i> , <i>mcl</i> A)
ZR1468	tctgcattacacttaactaat	
ZR1206	CGCGCTACCGCCGGTAATCAG	Used for verification of pZR2053, genes (<i>pcs</i> , <i>mcr</i>)
ZR1397	GCAAAGACTACGGGTGGATTG	
*lower cases indicate restriction site(s).		

5.2.4 Growth curve and Ethanol Production Experiment

To analyze growth rate and ethanol production of S4335, S4630, and S557 a growth experiment was conducted. All previously stated cultures were adjusted to an O.D._{700nm} of 0.4. After the O.D. was adjusted 100µL of culture was inoculated into 30mL of BG-11 with appropriate antibiotic as seed cultures. After seed cultures reached an O.D._{700nm} between 0.5 and 0.7 seed cultures were adjusted again to an O.D._{700nm} of 0.4. After all cultures were adjusted experiment cultures were inoculated using 100µL of adjusted seed culture to inoculate into 50mL of BG-11 with appropriate antibiotics. Strains used in the experiment included S4335 in both BG-11+ 100 µg/mL Nm. S4630 was inoculated into 50mL of BG-11 + 100 µg/mL Nm + 10 µg/mL Em. S557 was

inoculated into 50mL of BG-11 + 100 $\mu\text{g/mL}$ Nm. Schedule of samples taken were conducted as listed in Table 15. O.D. readings were performed using 300 μL of culture aliquot into a Costar 96 (Corning™ mfr. No. 3615) well plate and O.D. readings were taken at 700 nm using the Plate Reader (BioTek Synergy 2 Multi-detection Microplate Reader). Medium samples were taken as 1mL aliquots and placed into 1.5mL eppendorf tubes. Medium samples were processed by centrifugation at 12,000 x g for 10 min. Supernatant was then removed and placed into a fresh eppendorf tube and frozen overnight at -20°C. Samples were then thawed at room temperature and then centrifuged again at 12,000 x g for 10 minutes. Supernatant from the medium samples were then passed through a 0.2 μM sterile filter and placed in a 1mL HPLC vial for HPLC analysis.

Table 15: Sampling schedule for 3-HPA Bypass

Day 0
Day 2
Day 4
Day 6
Day 8
Day 10
Day 12
Day 14

5.2.5 HPLC Analysis

An Agilent HPLC (1220 Infinity II LC System) with a refractive index detector was used. Reaction products and standard compounds were detected by absorbance at 260 nm. A RHM-monosacchride H⁺ column was used (Rezex 7.8mm \times 250mm, Rezex) was equilibrated at a flow rate of 0.6 ml min⁻¹ with ddH₂O water buffer. An isocratic

program with a runtime of 25 min was used. Ethanol concentration was identified through the use of an ethanol standard curve and a retention time of 22 minutes.

5.2.6 Chlorophyll Extraction and Analysis of Photosynthetic Activity

Chlorophyll was extracted using a 90% methanol extraction procedure described previously (Meeks & Castenholz, 1971). Oxygen evolution was quantified in 1 mL culture samples that were normalized for O.D._{700nm} of 1.0 in BG-11 with 1mM NaHCO₃. Oxygen evolved was detected and analyzed using a Clark-type electrode and DW2 Oxygen Electrode Chamber with O₂ View Oxygen Monitoring software (Hansatech). Light was provided to the samples using an LS2/H Tungsten-halogen 50W light source and adjusted with neutral density filters. Light intensity and sample temperature was monitored using a Quantitherm light-temperature meter during experimentation. All measurements included three replicates to ensure accuracy.

5.3 Results

5.3.1 Expression and verification of 3-HPA genes

The six genes responsible for the photo-respiratory bypass *pcs*, *mcr*, *mcl*, *mct*, *meh*, and *mch* were cloned into two separate plasmids pZR2093 and pZR2053. Plasmid pZR2093 harbored *mcl*, *mct*, *meh*, and *mch* under a *glnA* promoter and ethanol producing genes *pdh* and *adhB* under a *psbA1* promoter with erythromycin resistance as the antibiotic selection marker. Plasmid pZR2053 harbored *mcr* and *pcs* under a nitrate inducible promoter, *pNir*, with neomycin resistance as the antibiotic selection marker. Presence of the two plasmids was verified using whole cell colony PCR of the conjugates

obtained on antibiotic selection plates (Figure 22, Figure 23, Figure 24). The resulting strain S4630 was cultured in BG-11 medium for subsequent experiments. Coomassie stain and western blot were performed on mutants bearing pZR2093+2053 plasmids (Figure 25 and Figure 26). It was found from the Coomassie stain that the two largest proteins were observed in pZR2093+2053 mutant that were absent in the pZR2011 mutant. Pcs and Mcr were the two largest proteins in the 3-HPA photorespiratory bypass that can be observed in the Coomassie stain.

Western blot was performed in addition to Coomassie stain. Western blot showed for presence of the myc-epitope tagged Mcr protein at 139 kDa. However, another protein mch was not observed which bared the myc-epitope as well.

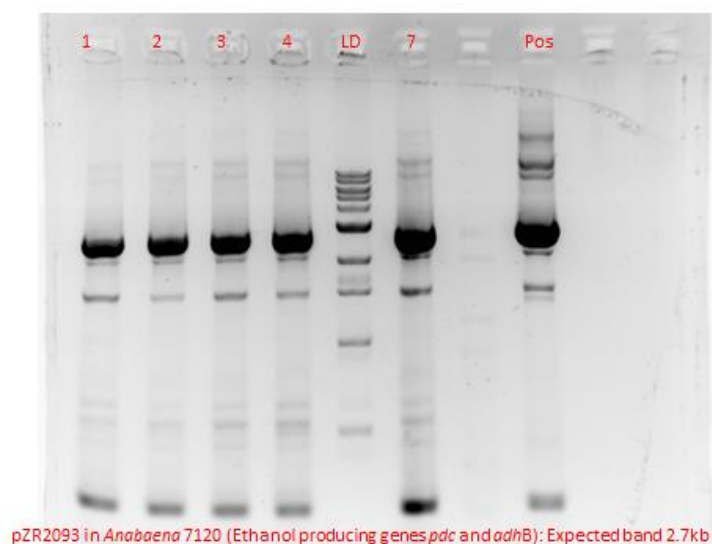


Figure 22: cPCR verification of pZR2093 genes *pdc* and *adhB* in *Anabaena* 7120.

Colony numbers 1-4 and 7 were all positive for the 2.7kb expected band. Positive control and ladder are listed as (Pos) and (LD), respectively.

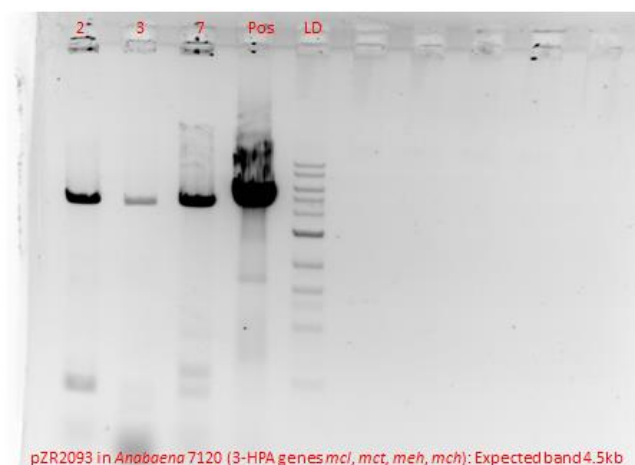


Figure 23: cPCR verification of pZR2093 genes *mcl*, *mct*, *meh*, and *mch* in *Anabaena* 7120 bearing pZR2093+pZR2053. Colonies 2,3 and 7 were all positive for the 4.5kb expected band. Positive control and ladder are listed as (Pos) and (LD), respectively.

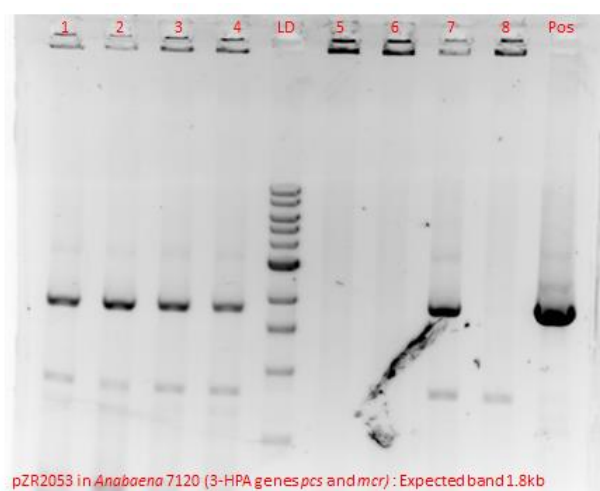


Figure 24: cPCR verification of pZR2053 genes *pcs* and *mcr* in *Anabaena* 7120 bearing pZR2093+pZR2053. Colonies 1-4 and 7 were all positive for the 1.8kb expected band. Positive control and ladder are listed as (Pos) and (LD), respectively.

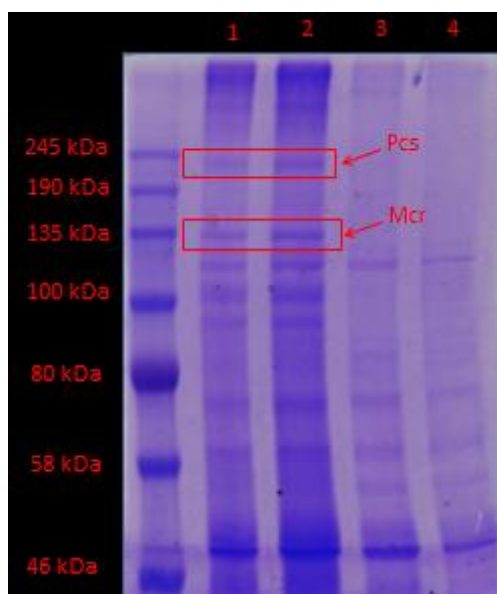


Figure 25: Coomassie stain of protein extracts from *Anabaena* PCC 7120 cultures. 1) pZR2093+ pZR2053, 10 µL protein extract. 2) pZR2093 + pZR2053, 15 µL protein extract. 3) pZR2011, 10 µL protein extract. 4) pZR2011, 15 µL protein extract. Two proteins observed Pcs and Mcr are present in pZR2093 + pZR2053 and absent in pZR2011.

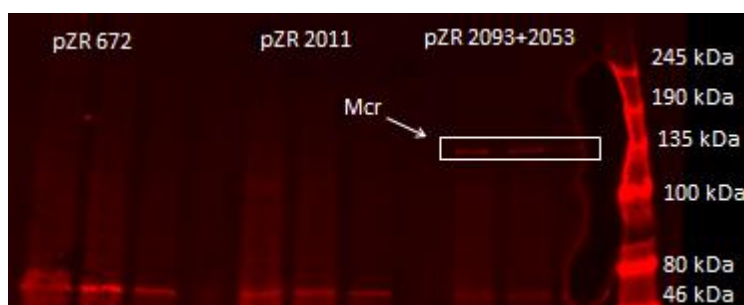


Figure 26: Western Blot of *Anabaena* PCC 7120 cultures. Protein extract amount was 15, 10, and 5 µL from left to right for each culture. Antibody for myc-epitope was used for visualization of the western blot. Protein bands observed at ≈ 46 kDa were believed to be non-specific binding.

5.3.2 Growth Experiment

Growth experiment was performed on two mutants (biological replicates #2 and #3) bearing pZR2093 and pZR2053. The growth experiment also included wild-type and a mutant bearing pZR2011 (Figure 27). Growth experiment showed best growth was achieved by wild-type followed by mutant bearing pZR2011 and lastly the mutants bearing 3-HPA bypass genes pZR2093 +2053 #2 and #3.

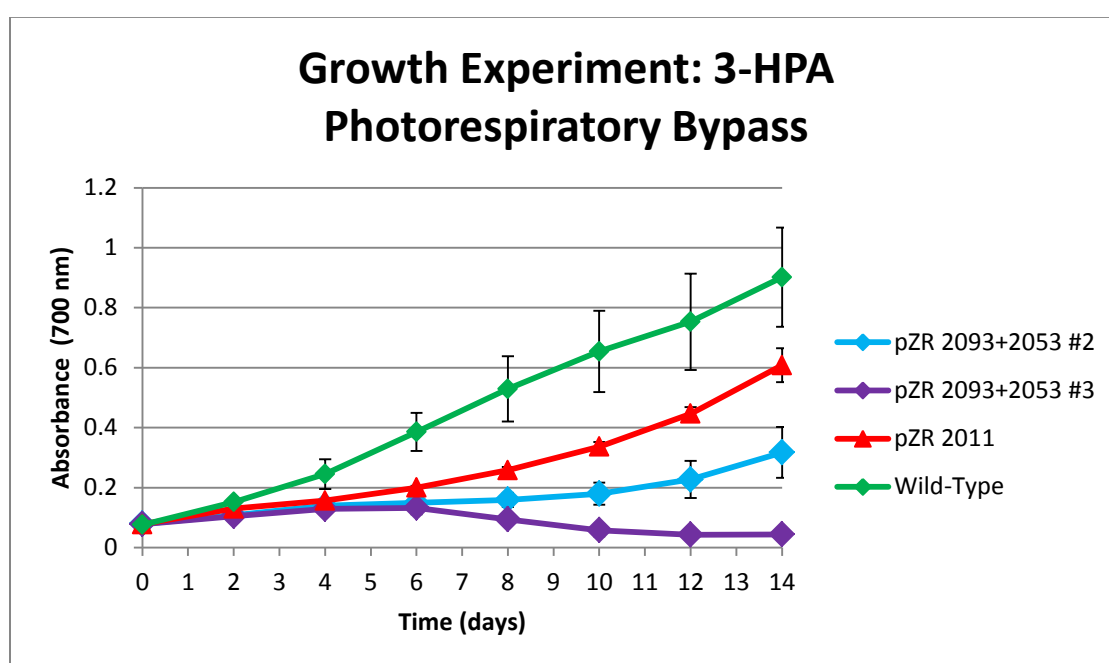


Figure 27: Growth experiment of 3-HPA bypass mutants. Growth experiment included two biological replicates pZR2093 +2053 #2 and #3 with another mutant pZR2011 and wild-type as control.

5.3.3 Photosynthetic Activity

Photosynthetic efficiency was calculated for all cultures included in the growth experiment. The cultures in the experiment included wild-type (WT), mutant bearing pZR2011, and biological replicates bearing pZR2093 and pZR2053 #2 and #3 (Figure 28).

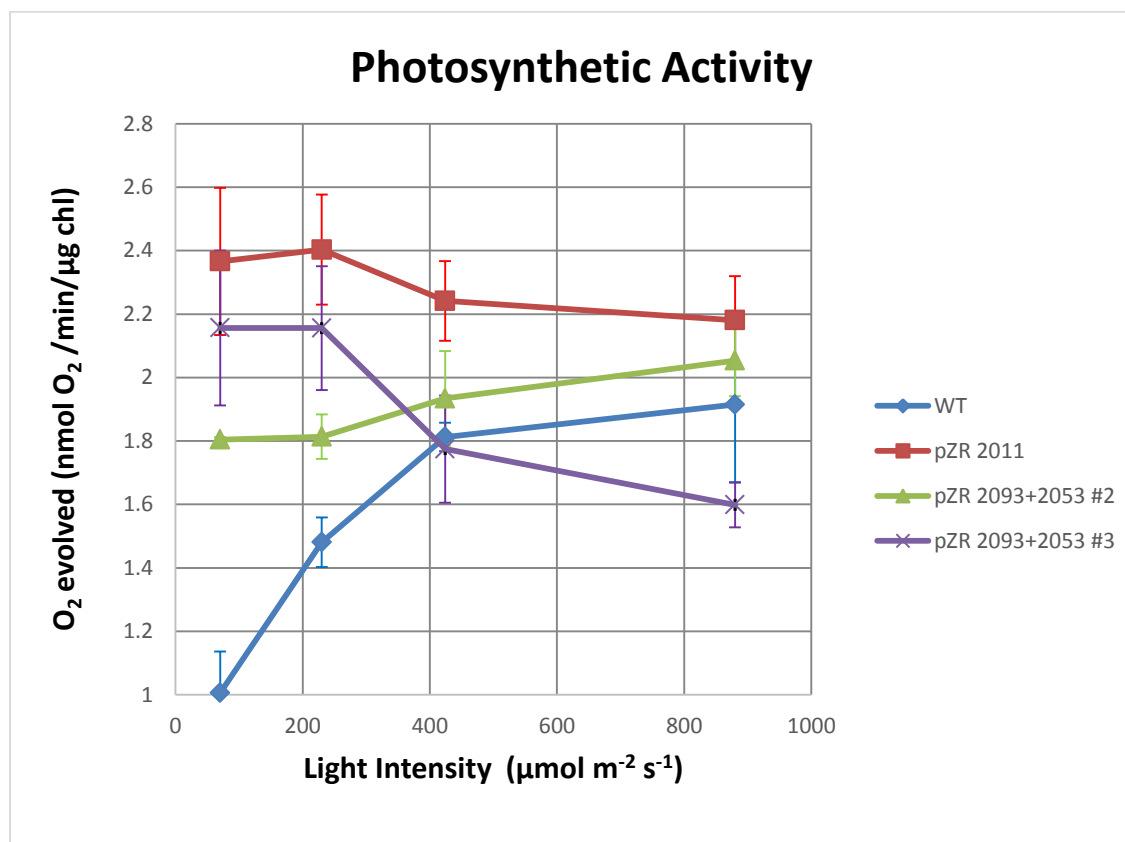


Figure 28: Photosynthetic activity of WT, mutant bearing pZR2011, and mutant bearing pZR2093 + pZR2053 biological replicates #2 and #3. All cultures were performed on species *Anabaena* PCC 7120. 1-mL aliquots (normalized at O.D.₇₀₀ at 1.0) in BG-11 + 1mM NaHCO₃ were measured for oxygen evolution in four different light intensities 70, 230, 424, and 880 μmol m⁻² s⁻¹.

5.3.4 Ethanol Production

Ethanol production was measured according to the directions dictated in materials and methods section. No ethanol production was detected in any of the samples collected. Standard curve was processed before each batch of samples to ensure that instrument error was not the cause for the absence of ethanol.

5.4 Discussion

In this study the synthetic CO₂ fixing photorespiratory bypass (3-HPA) was introduced into the photoautotrophic organism cyanobacteria *Anabaena* 7120. This pathway was introduced to only include the enzymes necessary to result in a net gain of carbon fixation while avoiding a net loss of fixed nitrogen. The addition of this synthetic pathway into *Anabaena* 7120 should allow for an additional carbon fixation cycle while the pathway itself is insensitive to oxygen. This allows a bypass to native carbon fixation that utilizes RubisCO which has poor specificity between O₂ and CO₂ which can lead to photorespiration and ultimately a loss of fixed carbon. The majority of metabolic engineering focuses on the introduction of metabolic pathways for anabolic production of target molecules such as those in biofuel producing pathways. This synthetic metabolic pathway is self-sustaining, in which glycolate/glyoxylate can be utilized by the pathway to fix carbon when available.

In previous studies the stoichiometrically correct values have been calculated for the formation of two glycolate molecules from each CO₂ molecule released in the C₂ cycle (Shih et al., 2014). It was determined that to reassimilate two molecules of glycolate the synthetic 3-HPA cycle would require 6 ATP equivalents and 4 NAD(P)H

which allows for the fixation of two additional molecules of bicarbonate. Bicarbonate is the inorganic form of carbon present in cyanobacteria. This pathway allows for the formation of fixed carbon without the net loss of fixed nitrogen NH_3 . It should be noted that if the pyruvate produced from the 3-HPA cycle is recycled into CB cycle (Calvin-Benson) two additional ATP equivalents per pyruvate would be required due to AMP forming pyruvate phosphate dikinase enzyme. Consequently, even with the additional ATP equivalent requirement of the recycled pyruvate the synthetic bypass would still compare favorably with the photorespiratory C_2 cycle present in cyanobacteria. In contrast, the aggregate of the C_2 cycle and CB cycle would require 11 ATP equivalents, 4 NAD(P)H, and 2 reduced ferredoxins to replenish the lost CO_2 and NH_3 as well as fix an additional 2 molecules of CO_2 to arrive at the same net carbon fixation as the synthetic 3-HPA pathway.

From the results obtained in this experiment it was found that the introduction of 3-HPA photorespiratory bypass did not yield better growth and only marginally increased photosynthetic activity. The expression of the genes involved in 3-HPA bypass showed that the genes contained on pZR2053 were adequately expressed from the western blot. It is not known for certain if the four genes involved in the 3-HPA bypass (*mch*, *meh*, *mct*, *mclA*) contained in pZR2093 were properly being expressed. It is believed that the gene containing the myc epitope *mch* was degraded during the protein extraction method and lead to the absence of this protein in the western blot. Growth data obtained from the experiment showed that there was no growth increase between wild-type and the mutant strains bearing the genes involved in the 3-HPA bypass. Photosynthetic activity experiment revealed that the mutant strain bearing the ethanol production genes had a

higher photosynthetic activity than wild-type. Mutant strain bearing plasmids pZR2093 and pZR2053 biological replicate #2 (S4630) showed only a marginal increase in photosynthetic activity. Strain 4868 showed a substantial decrease in photosynthetic activity that was reflected in the growth data as the culture decreased in O.D._{700nm} on day 6 and never recovered. Possible explanation for the poor performance on this mutant strain is likely that one or more genes were not expressing properly which lead to toxic intermediate build-up that greatly inhibited its ability to grow. A previous study of the addition of 3-HPA bypass has revealed that two toxic intermediates can be formed from the addition of the six genes responsible for the photosynthetic bypass (Shih et al., 2014). PCS and MCR are the last two proteins in the 3-HPA bypass and subsequently produce toxic intermediates propionyl-CoA and 3-hydroxypropionate (3OHP), respectively. The buildup of 3-HPA in the cell can lead to organic acid toxicity while propionyl-CoA can lead to the inhibition of pyruvate dehydrogenase and citrate synthase (Horswill, Dudding, & Escalante-Semerena, 2001). Either one or both of these toxic intermediates likely contributed to the decrease in growth rate observed in this study. It is interesting to note that although the growth rate of strain S4630 was much lower it showed that it was marginally higher in photosynthetic activity especially at lower light intensities. The higher photosynthetic activity in S4630 may mean that this biological replicate has the full functioning 3-HPA bypass but the toxic intermediates are hampering the growth rate.

Ethanol production from media samples were collected but no ethanol production was detected in the entirety of the study. Only traces amounts of ethanol were ever detected in S4335 and no consistent production was ever detected. This study attempted to improve the ethanol production by introducing the 3-HPA bypass in addition of the

genes *pdh* and *AdhB* responsible for the production of ethanol. However, since the introduction of the 3-HPA photosynthetic bypass was not stable to the mutant strains S4630 and S4868 which likely resulted in ethanol never being produced.

Various avenues could be explored to further study the introduction of the 3-HPA bypass. Improvements could be made by substituting genes *pcs* and *mcr* with mesophilic homologs since these genes were initially identified in *Chloroflexus* which is a thermophile. Both of these genes encode for very large proteins that likely impose a large amount of stress on the cells to properly synthesize and fold properly. Introduction of the same genes from a mesophilic homolog may allow for better expression of these genes.

Chapter 6: References

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